ISOLATION, CHARACTERIZATION, AND CHEMISTRY OF TAXANES FROM VARIOUS TAXUS SPECIES

By

JOHN STEVEN JUCHUM

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1999

This work is dedicated to Dr. Koppaka V. Rao, my advisor until his unexpected passing last year. I would also like to dedicate it to my parents, who have always shown great faith in their youngest son.

ACKNOWLEDGMENTS

Much of this work was done under the direction and guidance of the late Dr. K. V. Rao. I am thankful for all that he taught me and hope that I can continue the work that he started. I would like to thank Dr. Stephen Schulman for stepping in as my new advisor until this work was complete. I would also like to thank the other members of my committee--Dr. Kenneth Sloan, Dr. John Perrin and Dr. William Dolbier. I would like to thank Dr. Margaret James and Dr. Donghai Wu for their availability during my oral examinations. I would to thank the entire medicinal chemistry faculty for their support and dedication in seeing my work finished after Dr. Rao's passing. I would also like to thank Jan Kalman and Nancy Rosa for their assistance in the details of this endeavor.

I would like to thank my family and friends for their constant support and encouragement. Finally, I would like to thank Richard Davies, James Johnson, and Ravi Orugunty, who were my fellow students under Dr. Rao.

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ISOLATION, CHARACTERIZATION AND CHEMISTRY OF TAXANES
FROM VARIOUS TAXUS SPECIES

By

John Steven Juchum

December 1999

Chairman: Koppaka V. Rao
Major Department: Medicinal Chemistry

Paclitaxel is a potent anticancer agent isolated from the bark of the pacific yew (*Taxus brevifolia*) tree. It is one of a class of compounds, the taxane diterpenoids, which are found in all species of yew. All species and varieties of yew are potential sources of paclitaxel or other taxanes that may serve as precursors to semi-synthetic paclitaxel. This work deals with an efficient means of isolating paclitaxel. It also deals with the isolation and identification of many novel taxanes.

Most published methods of isolating paclitaxel and other taxanes rely on chromatography on a polar stationary phase (i.e., silica, Florisil or alumina). Such media tend to permanently retain some amount of the materials run through them leading to loss of important compounds. In addition, re-use of the stationary phase is limited. A chromatographic medium which does not have these limitations was investigated.

Chromatography on reverse-phase C-18 bonded silica, at low pressure, provides an efficient means of isolating important taxanes (including paclitaxel) from extracts of yew bio-mass. The reverse-phase medium does not retain important compounds. It can also be washed (in-column) for re-use over numerous iterations thus providing some cost advantage for the more expensive medium.

Reverse-phase chromatography, in large scale, was applied to chloroform extract solids of *Taxus brevifolia* bark. It gave a yield of paclitaxel more than four times that in previously published works. Twice the yield of paclitaxel from *Taxus* × *media* Hicksii needles was also achieved via this method. Reverse-phase isolation proved to be an efficient means of isolating taxanes.

Additional isolation work was necessary to provide some compounds in pure form. Continued isolations were performed on these two plant sources to identify new compounds. Several novel taxanes are described. Other taxanes were isolated for the first time from these plant materials.

Little work has been published on the heartwood of *Taxus brevifolia*. The heartwood was also processed by large-scale reverse-phase chromatography to determine its taxane constituents. The wood contains many interesting and potentially useful taxanes including paclitaxel C, 10-deacetylpaclitaxel C and 10-deacetylpaclitaxel C-7-xyloside.

CHAPTER 1
INTRODUCTION

A Brief History of Paclitaxel

Paclitaxel (Compound **1**, Figure 1.1), a potent anticancer agent, is a naturally occurring compound found in the bark of the pacific yew tree (*Taxus brevifolia*, Nutt.). Paclitaxel is a member of a class of compounds, the taxane diterpenoids, which are present in yew plants (Taxaceae). Paclitaxel is currently used in the treatment of breast and ovarian cancers and shows promise in the treatment of non-small cell lung cancer and head and neck cancer.[56]

Paclitaxel was discovered because of a research program which commenced forty years ago. In the late 1950s, The National Cancer Institute (NCI) started a program to screen natural compounds for activity against various forms of cancer. Collection of samples from higher plants began in 1960. In August 1962, Arthur Barkley, a botanist with the United States Department of Agriculture (USDA), collected specimens of the pacific yew tree in the Gifford Pinchot National Forest in Washington. An extract of *Taxus brevifolia* stem bark showed *in vitro* activity against 9KB cells (a cell line derived from human cancer of the nasopharynx) in early 1964.[62]

Monroe Wall at Research Triangle Institute (RTI) had requested to work on any extracts that exhibited 9KB activity. From previous work, Wall had observed a correlation between L1210 (mouse leukemia) *in vivo* activity and 9KB cytotoxicity. Use of the L1210 cell line eliminates the risk associated with the use of human cell lines like

9KB. He received 30 pounds of the bark later that year. Bioassay (L1210) guided fractionation of the extract yielded the active compound in 1966. Final isolation of the compound was achieved by June of 1967 and was reported at the national meeting of The American Chemical Society in Miami Beach, Florida, that year.[67] The structure of the compound, dubbed Taxol ("Taxol" is now the trademark of Bristol-Myers Squibb Co.), was published in the *Journal of the American Chemical Society* in 1971 by Wani et al.[68]

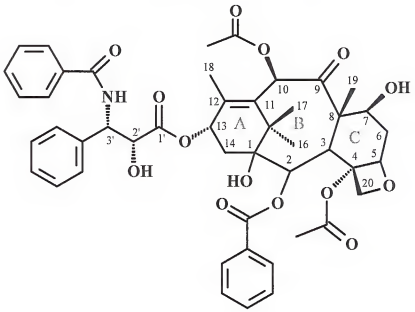


Figure 1.1. Paclitaxel (Taxol, Compound **1**)

Paclitaxel was present in low amounts in the bark and was very difficult to isolate. In the cell lines tested, paclitaxel was no more active than other anticancer agents were. The moderate activity and limited availability of paclitaxel caused it to sit on a shelf at the NCI for several years. In 1974, paclitaxel showed very good activity in a solid tumor assay against B16 Melanoma. Through the efforts of Matthew Suffness, paclitaxel was finally selected as a NCI development candidate in 1977.

The unique structure of paclitaxel gave hope of a new mechanism of action against cancer. This hope was dashed, albeit briefly, when Fuchs and Johnson discovered that paclitaxel inhibited cell proliferation at the G2-M phase of the cell cycle, blocking mitosis.[24] Paclitaxel appeared to be just one more compound in a series of naturally occurring spindle poisons. Only a few months later that hope was restored when the Horwitz group discovered that paclitaxel stabilized microtubules and promoted their polymerization rather than causing depolymerization of the microtubules as the known spindle poisons, such as vinblastine or colchicine, did.[58]

The uniqueness of both paclitaxel's structure and activity spawned a fury of research into paclitaxel on many fronts. As the amount of research into paclitaxel as a drug candidate grew, the limited availability of paclitaxel became more problematic. Efforts toward solving the "taxol supply crisis" included: total synthesis from readily available compounds, semi-synthesis from naturally occurring analogs of paclitaxel, isolation from another source (other *Taxus* species) and more efficient isolation from the bark of *Taxus brevifolia*.[19]

Meeting the Paclitaxel Supply Crisis

The pacific yew tree is a slow-growing tree found in old-growth forests of the northwestern United States and southwestern Canada. New companies sprang up to try to meet the increasing demand for paclitaxel. Lumber companies could now profit from trees which had no value in wood or paper production. The supply crisis was exacerbated by the concerns of environmentalists for the old-growth trees. Their concerns were both for the trees (depletion of the species) and for the endangered spotted owl whose habitat was in the old-growth forest.

Analysis of extracts of the bark by high precision liquid chromatography (HPLC) showed that far more paclitaxel (up to 0.069%) was present in the bark than could be isolated by current means.[66] The development of a more efficient means of isolating paclitaxel was one approach to solving the supply issue. The process developed by Dr. K. V. Rao could produce at least four times as much paclitaxel as other schemes. In addition to a much higher yield of paclitaxel, seven analogs of paclitaxel were also isolated in crystalline form. His process will be dealt with in Chapter 2.

Extracts of plant materials contain complex mixtures of compounds. An analysis of such mixtures may serve to identify new analogs of paclitaxel, which then could be converted to paclitaxel or a similar active compound. Chapter 3 is a continuation of the work covered in Chapter 2 where such a mixture (obtained in the process of isolating paclitaxel) is examined.

HPLC analysis was useful in identifying other species and varieties of yew plants that also contained paclitaxel. The needles of the ornamental hybrid *Taxus* × *media* Hicksii may serve as a new source of paclitaxel. According to HPLC analysis, the dry needles contain about 0.01%, by weight, of paclitaxel.[70] Though *Taxus brevifolia* bark has a higher concentration of paclitaxel, the trees must be felled to obtain the bark, whereas the *Taxus* × *media* Hicksii needles are a renewable resource. Work on the isolation of paclitaxel and other compounds from *Taxus* × *media* Hicksii needles will be covered in Chapter 4.

Taxus brevifolia trees have been harvested by the thousands to obtain the bark necessary in meeting the demand for paclitaxel. The bark is stripped from the trees and the wood is left as waste. The heartwood of the pacific yew is examined in chapter 5 to

determine what taxanes are present and whether the wood might be useful as a source of paclitaxel or other compounds of importance.

The European yew tree (*Taxus baccata*) does not contain an appreciable amount of paclitaxel, but in 1980, a compound (10-deacetylbaccatin III) was discovered in its needles, which could be converted to paclitaxel. The first of synthesis of paclitaxel from 10-deacetylbaccatin III (Compound **2**, Figure 1.2) was achieved in 1986.[28] Along the way, docetaxel (a semi-synthetic analog of paclitaxel) was synthesized in 1984.[60] At that time, docetaxel (Compound **3**, Figure 1.2) was known as Taxotere ("Taxotere" is the trademark of Rhone-Poulenc). Docetaxel has been developed as an anticancer agent in Europe. Docetaxel has a spectrum of activity similar to that of paclitaxel. The two compounds make up the newest class of anticancer agents, the taxane anticancer agents.

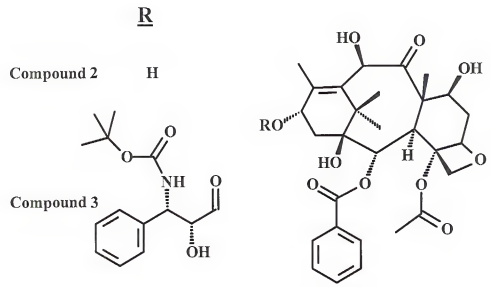


Figure 1.2. 10-Deacetylbaccatin III (Compound **2**)
And Docetaxel (Taxotere, Compound **3**)

The complexity of the compound paclitaxel made it an immediate target for synthetic organic chemists. At this time, five research groups have published total syntheses of

10-deacetylbaccatin III and so, consequently paclitaxel. Each group has taken a different approach to forming the taxane core of the compound. Though the complexity of paclitaxel makes total synthesis unfeasible as a source of the drug, the varied synthetic routes have other benefits. A greater understanding of the chemistry of paclitaxel aids in determining which parts of the molecule are responsible for its anticancer activity (Structure-Activity Relationship or SAR). Knowledge of the SAR of a drug can aid in the development of new drugs with different characteristics.[20, 30, 41, 44, 69]

A great deal of work has been done on the chemistry of paclitaxel and of other taxanes. Systematic studies on the order of reactivity of reactive sites in the taxane core are crucial in the development of syntheses. In Chapter 6, reactions are carried out on selected taxanes that have been isolated from plant materials.

CHAPTER 2
ISOLATION OF PACLITAXEL USING
REVERSE PHASE CHROMATOGRAPHY

Isolation of Paclitaxel

The first isolation of paclitaxel by Wall and Wani used Craig Countercurrent Distribution (CCD). This was an attempt to prevent the destruction (or permanent retention) of compounds which is inherent with chromatography on adsorbents such as silica or alumina. The plant material was first extracted with ethanol and concentrated. The extract was then partitioned between water, and chloroform and methanol (4:1). The chloroform layer was concentrated to dryness and placed in the first of three CCD systems. Fractions, showing activity, from the third CCD run were dried and triturated with benzene to give 0.5 g of paclitaxel from 12 kg of bark (yield: ~0.004%).[67]

CCD consumes a great deal of time and large volumes of solvent. Wani et al developed a simpler procedure in an effort to get a sufficient amount of paclitaxel to characterize. The plant material was extracted and partitioned as before. The chloroform layer was then subjected to three different chromatographic steps: first on Florisil, then on Sephadex LH-20 and a final silica column. The paclitaxel was then crystallized from methanol and water.[68]

The process developed by Polysciences, Inc. for large-scale isolation starts by extracting the dried bark of *Taxus brevifolia* with methanol. The alcoholic extract is then partitioned between water and dichloromethane. The CH_2Cl_2 layer is concentrated to

dryness and then stirred with a one to one mixture of hexane and acetone. The solid is filtered out and the filtrate is chromatographed on a florisil column with the same one to one mixture of solvent. The paclitaxel containing fractions are taken to dryness, crystallized from aqueous methanol and then recrystallized from acetone/hexane. The crude paclitaxel is then rechromatographed on silica with 1.5-3% isopropanol in dichloromethane. The yield of paclitaxel from the bark is approximately 0.01%.[32]

Reverse-Phase Processing

Reverse-phase chromatography as a means of isolating paclitaxel was first developed in small-scale under laboratory conditions by Dr. K. V. Rao.[48] A pilot plant was set up to test whether this method could be applied to large-scale isolation of paclitaxel. This method of isolation had a much greater yield of paclitaxel and with higher purity than other published isolations. This process also provided seven analogs of paclitaxel, each of which could be synthetically converted to paclitaxel. [49, 52] See Figure 2.1 for a comparison of processes.

The first part of the isolation procedure is the same as other methods: extraction of the dried bark in methanol, concentration of the extract, partitioning between water and chloroform and concentration of the chloroform layer to dryness. Here the processes diverge. The dried chloroform layer is placed directly on a C-18 reverse-phase chromatographic column in 25% acetonitrile/water. The column is eluted with a step gradient of 35-50% acetonitrile in water. Paclitaxel crystallizes directly from the column fractions. The crystals are filtered and recrystallized from acetone. A more detailed description of the process follows.

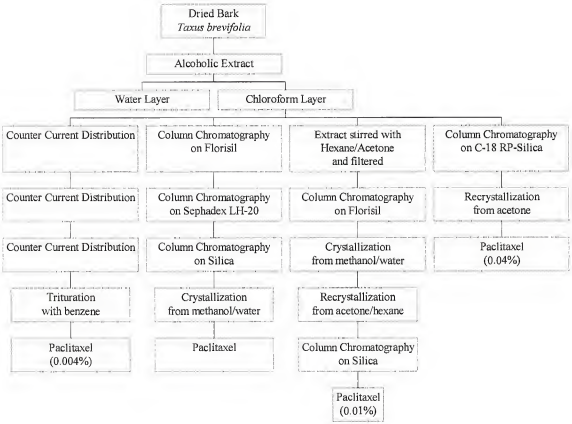


Figure 2.1. Process Comparison

The extraction of the bark was carried out as a batch process. The bark was placed in a stainless steel tank; approximately 200-260 lbs. of the bark could be extracted at a time in the 200-gallon tank and 300-400 lbs. of bark in the 300-gallon tank. Methanol was pumped into the tank with an air-driven pump. The methanolic extract was re-circulated through the bark for eight hours each day to facilitate the extraction. Three to four daylong extractions were carried out. The progress of the extraction was monitored by UV-absorbance values at 275 nm.

The methanolic extract was concentrated under reduced pressure (at 30°C) using a semi-continuously operated still with a receiving capacity of 220 gallons. Distillation was

carried out until the volume of the concentrate reached 20-25 gallons from the extract obtained from a 200-250 lb. batch of the plant material.

The concentrated methanolic extract was pumped from the still into a 100-gallon tank. The concentrate was stirred with water (10 gallons) and chloroform (20 gallons) for about 30 minutes using an air-driven stirrer. Methanol remaining in the concentrate tended to form emulsions with the chloroform layer. The first partition was allowed to stand overnight so that any emulsion might clear. The chloroform layer was drained off from the bottom into stainless steel containers. Two additional partitions were carried out with 15 and 10 gallons respectively of chloroform. UV-absorbance values were measured at 275nm to assure efficiency.

Concentration of the chloroform extract under reduced pressure was carried out in an all-glass, steam-heated, circulating evaporator. The concentrated chloroform extract was reduced further in a rotary evaporator to a thick syrup. The syrup was then poured into glass trays and converted to a powder form, using a vacuum oven maintained at 35-40°C. The powder (18-26 g per kg of the bark) was stored in tightly stoppered bottles at room temperature.

A chromatographic column, packed with C-18 bonded silica (15-35 μm), was equilibrated with 25% acetonitrile in water. A portion of the packing material was removed from the top of the column and was used to prepare the sample. The chloroform solids were first dissolved in acetonitrile, to which the equilibrated packing material was added. The mixture was stirred vigorously to keep everything in suspension. Three volumes (to one of acetonitrile) of de-ionized water was added slowly. The mixture was allowed to settle, giving two layers. The liquid layer was siphoned off and the remaining

slurry was poured into the column. The column was sealed and the liquid was pumped in using a diaphragm pump, which would not be harmed by any solids remaining in suspension. Two different sizes of column were used: a 4"x 4' stainless steel column that held 3-4 Kg of packing material and a 6" x 6' stainless steel column that held 12-13 kg. 500-700 g of the chloroform extract solids could be run on the 4" column and 2-2.5 kg on the 6" diameter column.

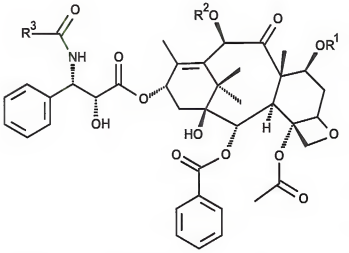
After the sample was pumped onto the column, the column was eluted with a step gradient of 35, 40, 45 and 50% acetonitrile in water. The change of solvent was dictated by TLC and HPLC analysis of the fractions (2 liters each). Generally, 40-50 liters of each solvent was used (for the 6" x 6' column). After this, the column was washed with methanol (20L), followed by 25% ethyl acetate in methanol (20L) and then 25% ethyl acetate/25% ligroin in methanol until the effluent was nearly colorless. Following this, the column was again washed with methanol and equilibrated with 25% acetonitrile in water.

The column fractions were allowed to stand at room temperature for 2-8 days, by which time, many of them showed a substantial degree of crystallization. The crystals were then filtered and put into groups defined by purity and composition using TLC and HPLC for analysis. Recrystallization from the appropriate solvent-system was the final step. Paclitaxel eluted with 50% acetonitrile/water, the last of eight taxanes to crystallize directly from the fractions.

The chromatography was also run using aqueous methanol as the solvent, starting with 30% methanol in water and continuing up to 65%. The results obtained were

comparable to those seen with the acetonitrile/water system, except that the rate and extent of crystallization of the various components was less.

The yield of paclitaxel from *Taxus brevifolia* bark, using this process, is 0.04% (at least four times the yield of other methods). The reverse-phase processing of the bark gives seven crystalline taxanes in addition to paclitaxel. 10-deacetylbaccatin III (Compound **2**, Figure 1.2) was obtained with a yield of 0.02% by weight of bark. The six other taxanes (Compounds **4-9**, Figure 2.2) are 10-deacetylpaclitaxel-7-xyloside (0.1%) (**4**), 10-deacetyl-paclitaxel-C-7-xyloside (0.04%) (**5**), 10-deacetylcephalomannine-7-xyloside (0.006%) (**6**), paclitaxel-7-xyloside (0.008%) (**7**), 10-deacetylpaclitaxel (0.008%) (**8**) and cephalomannine (0.004%) (**9**).[52]



	R^1	R^2	R^3
4	Xylosyl	H	C_6H_5
5	Xylosyl	H	C_5H_{11}
6	Xylosyl	H	
7	Xylosyl	Ac	C_6H_5
8	H	H	C_6H_5
9	H	Ac	

Figure 2.2. 10-deacetylpaclitaxel-7-xyloside **(4)**, 10-deacetylpaclitaxel-C-7-xyloside **(5)**, 10-deacetylcephalomannine-7-xyloside **(6)**, paclitaxel-7-xyloside (7), 10-deacetylpaclitaxel **(8)** and cephalomannine **(9)**

Paclitaxel and Analogs

Paclitaxel and seven analogs crystallize directly from the reverse-phase column fractions. Not all of the taxanes separate completely from others. After the crystals are

filtered out of the fractions, paclitaxel and four of the other taxanes can be purified further by recrystallization. The other three require a short silica column to obtain the pure compounds.

The order of elution, characterization, yields and other data on each of the eight taxanes are given below. In calculating the yields, it is to be noted that 100 kg of the bark generally gave 2.5 kg of the chloroform extract solids, which was applied to the column. Where relevant, the amount of the crude crystals obtained directly from the fractions, the amount after recrystallizations, and what was obtained by processing of the filtrates are given. The yields are based on HPLC analysis of the purified (97-99%) samples.

10-Deacetylbaccatin-III

10-Deacetylbaccatin III, which crystallizes almost immediately from the fractions, is the first taxane eluted from the column with 25-35% acetonitrile/water. It crystallized out of the fractions as glistening plates. The crude crystals (26g) were recrystallized from acetone twice to obtain colorless plates (18g). The filtrate was chromatographed on a silica column with chloroform, 2-5% acetone and 2-5% methanol in chloroform as the solvent system, followed by crystallization. This gave another 3g for a total of 21g (yield 0.02%). The proton and the carbon NMR spectra as well as the HPLC elution pattern were identical with those seen with an authentic sample. The melting point, 232-234°C, agreed with that in the literature.[11]

10-Deacetylcephalomannine-7-xyloside

Eluent fractions of 35-40 % acetonitrile/water gave 20 g of crude crystals. After filtration and recrystallization twice from acetone (with decolorizing charcoal), 6g of it was obtained as a colorless crystalline solid (0.006% yield) with a melting point of

250-252°C. The identity was confirmed by a comparison of the spectral data with those of a sample isolated and characterized earlier. [48]

10-Deacetylpaclitaxel-7-xyloside

A number of fractions eluting with 40-45% acetonitrile/water showed a heavy degree of crystallization. Analytical HPLC showed 10-deacetylpaclitaxel-7-xyloside to be the major constituent of these fractions. The fractions were filtered and the combined solid (180g) was recrystallized from a mixture of methanol and chloroform (1:1) with water (5-10%), using decolorizing charcoal. Recrystallization gave 99g (yield of 0.1%) of colorless rectangular plates with a melting point of 247-249°C. Its identity was confirmed by comparison with an authentic sample. 10-Deacetylpaclitaxel-7-xyloside is the major taxane component of the bark and was isolated in yields of 0.06-0.1%. The bark from the more mature trees approached the 0.1% mark, while the bark from younger trees was closer to the 0.06% range. Quantitative HPLC analysis of the bark extract showed that the content of this component was in the range of 0.12-0.14%, thus indicating a 70-80% efficiency of recovery.

10-Deacetylpaclitaxel-C-7 xyloside

This was obtained from the fractions eluted with 45 % acetonitrile. Using analytical HPLC, those fractions showing the highest ratio (8:1 or higher) for 10-deacetylpaclitaxel-C-7-xyloside to 10-deacetylpaclitaxel-7-xyloside were filtered and the combined solid dried (98g). After two crystallizations (charcoal) from the solvent-system described above, 40g (0.04% yield) of 10-deacetylpaclitaxel-C-7-xyloside was obtained as a

colorless crystalline solid with a melting point of 218-220°C (lit. 215-217). The proton NMR spectral data also agreed with those described by Sénilh et al. [59]

Paclitaxel-7-xyloside and 10-Deacetylpaclitaxel

The filtrates from the crystallizations of the 10-deacetylpaclitaxel-C-7-xyloside contained paclitaxel-7-xyloside and 10-deacetylpaclitaxel as the major components. These filtrates were combined with the other column fractions containing the same components, and concentrated to dryness. The solid (55g) was applied to a 500g silica column in chloroform. The elution sequence was 2% acetone (1L), 5% acetone (1.5L), 2% methanol (1.5L), 5% methanol (1.5L) and 10% methanol in chloroform (2L). The major components, 10-deacetylpaclitaxel and paclitaxel-7-xyloside, appeared in the 2% and 5% methanol in chloroform eluates respectively. The appropriate fractions were concentrated to dryness and the compounds were crystallized. 7.8g (0.008% yield) of paclitaxel-7-xyloside were obtained from acetone as a colorless crystalline solid with a melting point of 235-237°C. 8g (0.008% yield) of 10-deacetylpaclitaxel were crystallized from aqueous acetonitrile as a colorless crystalline solid (mp. 194-196°C). The spectral data agreed with those described in the literature. [48, 59]

Cephalomannine

At 45-50% acetonitrile/water, cephalomannine and paclitaxel co-elute. The fractions were analyzed by analytical HPLC to determine the relative composition. Crystals, from those fractions containing 10% or more of cephalomannine, were filtered and grouped separately. The solid (25 g) was purified by reverse-phase column chromatography using C-8 silica gel as the adsorbent and 40-45% acetonitrile/water as the eluent. The fractions

were monitored by HPLC. Crystalline solids, which separated out, that were essentially cephalomannine, were filtered and recrystallized once from acetonitrile/water and again from acetone/ligroin. 4g (0.004% yield) of cephalomannine were obtained as colorless needles with a melting point of 185-188°C. The analytical, physical and spectral data were identical with those described by Miller, et al. [40]

Paclitaxel

The crude crystalline solid (110g) from the fractions containing less than 5% cephalomannine was filtered and subjected to one of three alternative procedures. The first was to recrystallize it (using decolorizing charcoal) two or three times to reach final purity. Alternatively, decolorization was achieved by filtration through a short column of Florisil (50g for 10g of the sample) in chloroform. Washing with 1-2% methanol in chloroform eluted the paclitaxel, which was recovered and crystallized twice from acetone/ligroin. The paclitaxel so obtained was essentially free from cephalomannine (0.3% or less), as shown by analytical HPLC. The third alternative, for further purification (i.e. to remove the residual cephalomannine completely), was to subject the sample to ozonolysis in chloroform/methanol (9:1) at –70°C (acetone/dry ice bath).

In a typical experiment, 30g of the crude crystalline solid, obtained directly from the reverse phase column, was dissolved in the chloroform/methanol mixture (300ml), cooled to –70°C and saturated with ozone over a period of 45 minutes. After checking for completion of the reaction by HPLC, the reaction mixture was treated with dimethyl sulfide (10ml) to decompose the ozonide(s). The mixture was allowed to rise to room temperature and stand overnight. The mixture was concentrated to remove most of the solvent and then partitioned between water and chloroform; the organic layer was

separated and the extraction repeated twice more. The combined chloroform layer was concentrated to dryness and applied to a silica column (300g, 235-425 mesh) in chloroform. Elution with 5% acetone in chloroform gave the bulk of the paclitaxel, which was crystallized from acetone/ligroin, to give 12g of pure paclitaxel (0.04% yield). HPLC analysis showed this sample to have a purity of 99% or better, and to be free from cephalomannine and 7-epi-paclitaxel. Its physical, analytical and spectral properties were identical with an authentic sample obtained from the National Cancer Institute.

Conclusion

The current production of paclitaxel for clinical use takes two forms. Isolation from plant materials (various species) is still a main means of production in much of the world. Semi-synthesis from 10-deacetylbaccatin III has become the major source in the United States (Bristol-Myers Squibb) but a problem has arisen with patent infringement against Rhone Poulenc, the French pharmaceutical company which holds the patent. Since direct isolation remains a major, and competitive, source of the drug, improved methods of isolation still play a critical role. This is also true in the isolation of 10-deacetylbaccatin III or other semi-synthetic starting materials.

10-Deacetylbaccatin III had only been isolated from *Taxus brevifolia* in very low yield (<0.0003%) by standard-phase processing,[32] but Rao's process increased the yield (0.02%) by nearly two orders of magnitude. This compound is abundant in the needles of the European yew (*Taxus baccata*). Improved isolation over standard methods could greatly increase the availability of this important compound, which is currently used to make paclitaxel and the semi-synthetic drug docetaxel.

Each of the seven analogs, isolated by reverse-phase processing, may serve as a source of paclitaxel. Paclitaxel-7-xyloside can be converted directly to paclitaxel by hydrolysis of the xyloside moiety. 10-Deacetylpaclitaxel-7-xyloside (the most abundant taxane in the bark) can likewise be converted to paclitaxel by hydrolysis of the xyloside moiety and acetylation at the 10-position.[47]

The rest of the compounds can also be converted to paclitaxel. The compounds can be converted to baccatin III or 10-deacetylbaccatin III by removal of the side-chain and hydrolysis of the xyloside (if necessary).[39] More direct means of conversion are also possible. Use of Schwartz reagent converts the amide in the side-chain (e.g., cephalomannine or paclitaxel C) to a free amine, which then can be benzoylated to make paclitaxel. [42]

CHAPTER 3
TAXANES FROM THE BARK OF *TAXUS BREVIFOLIA*

Introduction

Large-scale processing of the chloroform extract of *Taxus brevifolia* bark (Chapter 2) yielded eight crystalline taxanes, including paclitaxel. Many other taxanes are present in the plant materials and so in the chromatographic fractions. Identification of these taxanes and any other compounds present expands the pool of knowledge concerning the phytochemistry of yews. Many reviews have been published on the phytoconstituents of yews, but much work is left to be done.[1, 35, 46] Such work is important in finding new taxanes, which might show activity, or be converted to paclitaxel or another active analog. For example, the discovery of 13-acetyl-9-dihydrobaccatin III, in *Taxus canadensis*, led to the semi-synthesis of the active analog: 9-dihydropaclitaxel.[29, 36] Regardless of activity, new taxanes can aid in determining the structure-activity relationships (SAR) of taxane anticancer agents by providing new compounds with modest structural differences.

Paclitaxel is the last of the eight compounds which crystallize directly from the eluate to leave the reverse phase column. The fractions, which eluted immediately after paclitaxel, were examined for content. Re-chromatography of these fractions yielded fifteen taxane constituents, many of which were obtained in crystalline form. [54]

Isolation of Taxanes

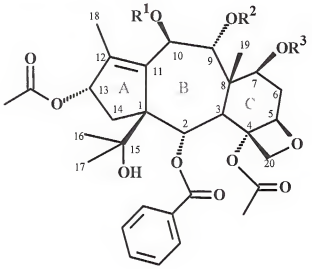
The first step in isolating compounds from the post-paclitaxel fractions was to identify target compounds to be isolated. Thin layer chromatography (TLC) is the most useful analytical method to do this. TLC (silica) run in a solvent system of 3% methanol and 10% acetone in dichloromethane gives paclitaxel a R_f of about 0.5. TLC slides are viewed under UV light (254nm) and then developed by spraying 1N H_2SO_4 on the TLC and charring on a hotplate. A spot on the TLC, which ran faster than paclitaxel, was observed both under UV (indicating the possible presence of a benzoate or cinnamate moiety) and by charring. Upon charring, this spot turned dark green. This spot resolved into several spots using a slower solvent system. In 8-10% acetone in dichloromethane the TLC shows three green spots on developing.

An initial chromatographic column was run to effect gross separation of the components of the post-paclitaxel fraction. Subsequent columns were then run to further purify the compounds. Several solvent systems for running the columns were tested to find an efficient means of separating the three target compounds. It was discovered that different solvent systems caused the elution order of compounds to change and so various systems were used throughout the course of this study.

Chromatography of the post-paclitaxel fractions on silica using increasing amounts of acetone (15-50%) in ligroine (hexanes) as the mobile-phase gave fair initial separation but ran slowly and used a large volume of solvent. Dichloromethane (CH_2Cl_2) proved to be a much better solvent for chromatography on silica.

The post-paclitaxel fractions were dissolved in CH_2Cl_2 and placed on a column with a ratio of 1g of sample per 10g of packing material (silica). The column was eluted with

CH_2Cl_2 with increasing amounts of acetone and methanol, while monitoring with TLC. The first and second of the target compounds eluted quickly with CH_2Cl_2 as the mobile-phase. The third green spot eluted with 2-5% acetone. A fourth compound showing a green spot on TLC eluted with 10% acetone. 10% acetone plus 10% methanol in CH_2Cl_2 eluted paclitaxel.



	R^1	R^2	R^3
10	Bz	Bz	Ac
11	Bz	Ac	Ac
12	Ac	Ac	Bz
13	H	Bz	Bz
14	Ac	Bz	Bz
15	H	Ac	Bz
16	Bz	Ac	Bz
17	Bz	Bz	H
18	Bz	Bz	Bz

Figure 3.1. 11(15→1) abeotaxanes

The fastest moving of the green spots, Compound **10** (Figure 3.1), crystallized from some of the fractions eluting with CH_2Cl_2. A second column using CH_2Cl_2 and up to 5% acetone as the mobile-phase gave total separation of spot one from spot two. Ethyl acetate in ligroine used as the mobile-phase reversed the elution order of the two spots. The second spot (acetone/CH_2Cl_2) was an amorphous solid. ^{1}HNMR spectroscopy showed it to be a mixture of two compounds. The two compounds (**11** and **12**, Figure3.1) proved to be isomers that can be separated by repeated crystallization from methanol or by chromatography on silica using an ethyl acetate in dichloromethane mobile phase.

Compounds **10-12** were previously isolated from *Taxus brevifolia*, but their structures were incorrectly assigned. The structures were later revised by the same group.[15-16]

Compound **10** (taxchinin C) was also isolated from *Taxus chinensis* (Chinese yew). This was one of four compounds reported by Fuji et al which are taxanes having a rearranged A-ring. The rearranged taxanes, 11(15→1) abeotaxanes, have a 5-7-6 membered ring system rather than the 6-8-6 taxane ring system seen in paclitaxel.[25]

The third target identified by TLC, compound **13**, crystallized from some fractions eluted with 2-5% acetone. Although crystalline, compound **13** gave a ^{1}HNMR spectrum (in $CDCl_3$) with broad, rounded peaks. At –20°C, the peaks resolved into two sets of peaks indicating the presence of two conformers in solution. Two sets of peaks were also observed in deuterated DMSO at room temperature.[54]

Compound **13** was identified by identification of reaction products. The compound was first acetylated with acetic anhydride and pyridine. The product acetate (Compound **14**, Figure3.1) was identifiable by NMR and was previously unknown. The presence of three acetate moieties in the product left uncertainty about the position of the hydroxyl on the parent compound. The compound was oxidized with Jones' Reagent in acetone. The resulting product, compound **19** (Figure 3.2), had a ketone with a ^{13}CNMR peak at 190ppm indicating conjugation with the 11,12-double bond. The ketone was determined to be at the 10-position rather than the 13-position by proton shifts in the ^{1}HNMR spectrum. Thus, the compound possessed a hydroxyl at the 10-position. Compound **13** was also unknown.

^{1}HNMR data for compound **13** was compared with that in published literature and with a compound (taxiflorine) isolated in this lab from *Taxus floridana*. It was seen that

abeotaxanes that have a hydroxyl at the 10-position and a benzoate ester at the 9-position all exhibit broad peaks in the ^{1}HNMR spectrum (at room temperature in $CDCl_3$). Another novel compound (Compound **15**, Figure 3.1) was isolated having a hydroxyl at the 10-position and an acetate at the 9-position. It did not show such band broadening. In each case, resolution of the broad bands into multiple peaks was seen at lower temperatures or in polar solvents. Compound **15**, like compound **13**, was also oxidized with Jones' reagent to give compound **20** (compare to compound **19**, Figure 3.2).[53, 63]

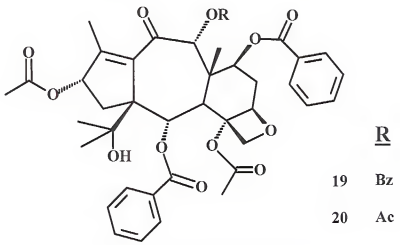


Figure 3.2. Jones' Oxidation Products

Further processing of the post-paclitaxel fractions yielded compound **15** and two more naturally occurring abeotaxanes (Compounds **16** and **17**, Figure3.1). Both were previously unknown. There were complications in isolating compound **16**, which will be discussed later. The fourth green spot on TLC, mentioned above, corresponds to compound **17**. Compound **18** was not isolated. It is the benzoylation product of compound **13**, and of compound **17**.

Fractions from the silica column, which preceded compound **10**, showed a spot on TLC that turned dark-blue upon charring with 1N H_2SO_4. This spot was isolated but

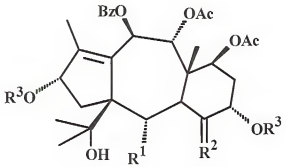
^{1}HNMR showed it to be a mixture. The mixture was dissolved in methanol to apply it to a preparative TLC plate. Here serendipity lent a hand as crystals started to form in the solution. The crystalline compound proved to be taxinine J (Compound **21**, Figure3.4).[71]

After taxinine J was crystallized and filtered out, the mother liquor was checked again by ^{1}HNMR. It remained a mixture, but it was possible to determine the presence of two compounds (neither of which was taxinine J). Three doublets at 4.17 ppm (7.8Hz), 4.54ppm (7.8Hz) and 5.02ppm (7.2Hz) are indicative of an oxetane ring. Two singlets at 4.72ppm and 5.15ppm and a broad singlet at 5.24 show the presence of a 4(20)-exocyclic double bond.

Selective ozonolysis was performed on a portion of the mixture to cleave the exocyclic double bond without cleaving the 11,12-double bond. The mixture was dissolved in methanol and dichloromethane and put in an acetone and dry ice bath. Ozone (from an ozone generator made by Ozone Research and Equipment Company, Phoenix, AZ) was bubbled through the solvent for thirty minutes. The solution was pale blue, which indicates saturation by ozone. Dimethyl sulfide was added to reduce any remaining ozone and the reaction mixture was allowed to return to room temperature and stand for two hours. Preparative TLC on the reaction product gave two bands visible under UV light (254nm). The bands were scraped off the plate and eluted with 10% methanol in dichloromethane. The faster band gave a crystalline compound (**16**). The slower band was eluted to give a new compound (Compound **22**, Figure3.3) with a ketone at the 4-position.

The parent compound (Compound **23**, Figure3.3) from which **22** was made was unknown. Compound **23** appeared to be a new analog of brevifoliol (Compound **24**,

Figure3.3). Brevifoliol is also an 11(15→1) abeotaxane but with a 4(20)-exocyclic double bond instead of an oxetane ring. The structure of brevifoliol was also first reported incorrectly and then revised.[3, 17, 26] Brevifoliol is present in the bark of *Taxus brevifolia* but not in the fractions analyzed here. It elutes from the reverse-phase column between 10-deacetylbaccatin III and 10-deacetylcephalomannine-7-xyloside but does not crystallize from the fractions.



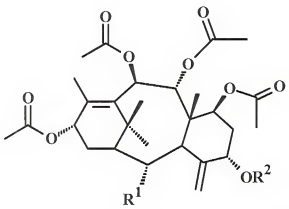
	R^1	R^2	R^3
22	OBz	O	Ac
23	OBz	CH_2	Ac
24	H	CH_2	H

Figure 3.3. Brevifoliol and Analogs

It was necessary to isolate compound **23** to confirm its proposed structure. Numerous solvent systems were tested on TLC to find one that would separate the two components. 10% acetone in benzene gave separation on TLC. For column chromatography (on silica), 1% acetone in benzene was used as the mobile phase. A HMBC (Hetero-Multi-Bond Correlation) spectrum was run to confirm the placement of ester groups on the structure. In this spectrum, the carbonyl carbon atom of an ester moiety will couple with the proton(s) (if any) on the carbon atom in the taxane backbone. The HMBC spectrum confirmed the proposed structure of compound **23**.

Column fractions and mother liquors from crystallizations were pooled into groups according to TLC behavior. Column chromatography on the pooled material gave several other compounds. Most of these other compounds isolated from the post-paclitaxel

fractions are members of the 4(20),11-taxadiene group of taxanes, having the 6-8-6 ring system.



	R^1	R^2
21	OAc	Cinn.
25	H	Cinn.
26	H	Ac
27	OAc	Ac

Figure 3.4. 4(20),11 Taxadienes

In addition to taxinine J (Compound **21**), three other compounds with a normal pattern of oxygenation were found (Figure3.4). These are 2-deacetoxytaxinine J (Compound **25**), 5α,7β,9α,10β,13α-pentaacetoxytaxa-4(20),11-diene (Compound **26**) and 2α,5α,7β,9α,10β,13α-hexaacetoxytaxa-4(20),11-diene (Compound **27**).[21, 35] Two other compounds with oxygenation at the 14-position were found. These are yunnanxane (Compound **28**, Figure 3.5) and a related compound (Compound **29**, Figure 3.5).[38]

A small quantity of baccatin VI (Compound **30**, Figure3.6), which has an oxetane ring, was also isolated. It is a noteworthy compound because it can be used as a starting material to make active analogs of paclitaxel. See the flowchart (Figure 3.7) for the order in which these compounds were isolated.

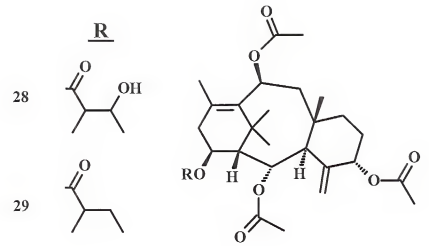


Figure 3.5. Yunnanxane (Compound **28**) and analog (Compound **29**)

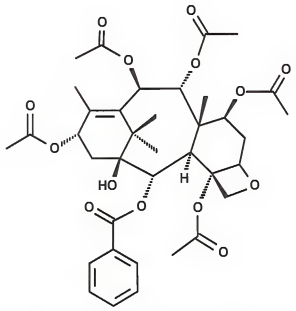


Figure 3.6. Baccatin VI (Compound **30**)

Characterization of Compounds

The structures of compounds were determined primarily using various NMR techniques (i.e., ^{1}HNMR, ^{13}CNMR, COSY, HETCOR, APT, DEPT and HMBC). Many of the compounds isolated were known and the acquired data compared with that

published in literature. Some compounds were identified by analogy to similar known compounds. Where applicable, unknown compounds were synthetically converted to other compounds (known and unknown) for identification. NMR and other characterization data are listed below.

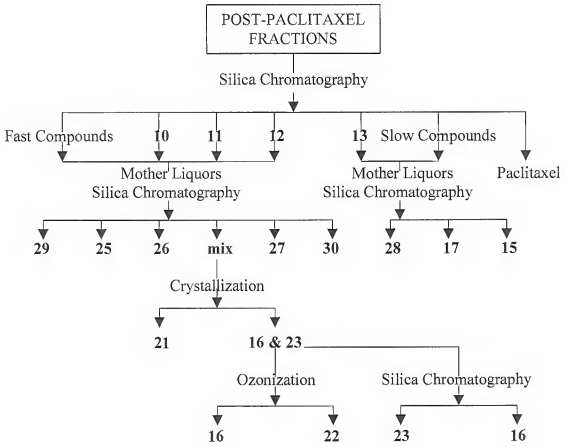


Figure 3.7. Isolation of Taxanes

Compounds 10-12

^{1}HNMR and ^{13}CNMR data for these compounds were found to agree with that published by Chu et al.[16] The structures assigned to the compounds were based on the

structure of baccatin VI (six-membered A-ring). Based on X-ray crystallographic studies of brevifoliol (and similarities in NMR), they reassigned structures for **10-12** based on a 5-membered A-ring, i.e. with the 11(15→1)-abeotaxane skeleton.[15] They had isolated the compounds from *Taxus brevifolia* bark as amorphous solids and so could not do X-ray analysis directly on them. Nor could they publish melting point data.

Before Chu et al had revised their structure assignments, Fuji et al reported the isolation of **10** from *Taxus chinensis* needles as a crystalline solid, which they named taxchinin C.[25] They correctly identified the compound as having a 5-membered A-ring. This was the first instance of a naturally occurring compound shown to have a 5-7-6-ring system. Such a structure had been seen previously only as a reaction product of paclitaxel when treated with electrophilic reagents.

The main identifying feature of the 11(15→1)-abeotaxane skeleton is a peak in its ^{13}CNMR spectrum. The C-1 carbon, a quaternary carbon, is located far downfield in the 5-7-6 membered ring system from where the C-15, quaternary carbon comes in the 6-8-6 membered ring system. The C-1 comes at ~68ppm where the C-15 comes at ~43ppm.

Compound **10** (taxchinin C) was obtained as a crystalline solid, mp 208-210°C (lit. 212-214°C [25]). The spectral properties agreed with those reported in the references.[16, 25]

Compounds **11** and **12** were obtained as a mixture. They could be separated by silica chromatography with ethyl acetate in dichloromethane.[16] Alternately, compound **12** crystallizes from methanol concentrating **11** in the filtrate.

Compound **11** was obtained as a colorless crystalline solid from ether, mp 210-212°C. Calculation for elemental analysis for $C_{42}H_{48}O_{14} \bullet H_2O$ was C: 63.47 and H: 6.34. Found: C: 63.82 and H: 6.38.

Compound **12** was obtained as colorless prisms, mp 203-205°C. The yield of **12** was 0.01% of bark, giving sufficient quantities to run reactivity studies. The spectral data agreed with those given by Chu et al.[16]

Compound 13

The ^{1}HNMR spectrum of compound **13** gave rounded peaks in $CDCl_3$ at room temperature (see Figure). The ^{13}CNMR spectrum was unintelligible. The ^{1}HNMR spectrum run in deuterated DMSO resembled that of a taxane but too many peaks were present. Since **13** was crystalline, i.e., a single compound, then the NMR spectra must indicate the presence of a rotameric equilibrium in solution. The spectrum taken at –20°C confirmed the presence of two conformers in a 2:3 ratio.

Such behavior has been observed in similar taxanes by other groups as well as having been observed in this lab on another compound (taxiflorine) that was isolated from *Taxus floridana*.[51, 53] Taxchinins I and J isolated by Tanaka et al, taxayuntin F isolated from *Taxus yunnanensis* by Yue et al, taxiflorine and compound **13** all exhibit such NMR spectra.[63, 73] All of these compounds share a structural feature. They are all 11(15→1) abeotaxanes having a hydroxyl at C-10 and a benzoate at C-9. 11(15→1) abeotaxanes having a hydroxyl at C-10 and an acetate at C-9, however, do not show such spectra. Examples of this are compound **15** (see below), a compound (same as taxacustin) reported by Chattopadhyay et al and taxayuntin E isolated by Yue et al.[9, 73] Another compound, isolated in this lab from *Taxus brevifolia* bark, having a 9-hydroxy and 10-benzoate structure gives a normal spectrum.[50]

The structure of compound **13** was determined by way of reaction products. Those are the acetylation product (Compound **14**), the benzoylation product (Compound **18**) and the Jones' oxidation product (Compound **19**).

Compound **13** was obtained as a crystalline solid from acetone-ligroine, which starts to decompose at 255°C. Specific rotation $[\alpha]_D$ in CH_2Cl_2 was 21°. IR (KBr, cm^{-1}): 3400, 3340, 1740-1710, 1595, 1575, 1485, 1470, 1450, 1370, 1310, 1270,1240,1170,1110, 1065, 1020,990,930,900,700,680. Calculation for elemental analysis for $C_{45}H_{48}O_{13}$ was C: 67.83 and H: 6.07. Found: C: 68.07 and H: 6.39.

Compound 14

A solution of compound **13** (0.1g) in pyridine (0.5ml) was treated with acetic anhydride (2 ml) and the mixture was left standing for 16 hours. After dilution with water and filtration, the acetate was chromatographed (CH_2Cl_2/ligroine) and obtained as a white amorphous powder. ^{1}HNMR values (δ) are: 1.17 (s, 3H, Me); 1.17 (s, 3H, Me); 1.56 (s, 3H, Me); 1.98 (s, 3H, Me); 2.0 (m, 2H, H-6 and H-14); 2.19 (s, 3H, Me); 2.20 (s, 3H, Me); 2.44 (dd (13.8Hz and 6.9Hz), 1H, H-14); 2.56 (br s, 1H, OH-15); 2.78 (m, 1H, H-6); 3.21 (d (6.9Hz), 1H, H-3); 4.21 (d (7.5Hz), 1H, H-20); 4.56 (d (7.5 Hz), 1H, H-20); 5.74 (br t, 1H, H-13); 5.89 (br t, 1H, H-7); 6.49 (d (10.8Hz); 1H, H-9); 6.53 (d (6.9Hz), 1H, H-2); 6.61 (d (10.8Hz); 1H, H-10); and 6.86, 6.88, 6.91, 7.19, 7.21ppm, 7.23ppm, 7.37, 7.39, 7.41, 7.47, 7.50, 7.52, 7.60, 7.63, 7.65, 7.80, 7.92, 8.03 and 8.06 (15H, Ar-H). ^{13}CNMR values (δ) are: 11.9, 13.2, 20.5, 21.1, 22.0, 25.1, 27.6, 27.6, 34.9, 36.8, 44.1, 44.5, 68.0, 68.4, 70.9, 74.5, 77.4, 78.7, 79.1, 84.6, 127.8, 128.0, 128.6, 129.6, 129.9, 130.5, 132.6, 133.5, 136.2, 147.2, 165.2, 165.7, 166.4, 167.9, 169.0, 170.6. Calculation

for elemental analysis for $C_{47}H_{50}O_{14} \bullet 0.5\ H_2O$ was C: 66.58 and H: 6.06. Found: C: 66.55 and H: 6.32.

A long-range HETCOR (HMBC) spectrum was run to aid in determining the placement of acetate and benzoate moieties on the taxane backbone. One of the benzoate carbonyl (Ph-CO) signals (δ 165.2) interacted with that of H-7. The second signal of Ph-CO (δ 165.7) showed an interaction with that at δ 6.5, where the chemical shifts of H-2 and H-9 overlap. The third Ph-CO (δ 166.4) did not show an interaction. Comparison with known compounds placed a benzoate on C-2 and acetates on C-4 and C-13. The final assignments for the C-9 and C-10 positions was made from the Jones' oxidation reaction on compound **13** (cf., compound **19**).

An interesting interaction was observed for one of the acetate carbonyls (δ167.8). It showed connectivity with the methyl (CH_3) signal at δ1.56. The normal acetate methyl comes in the 2.0-2.2ppm range.

Compound 15

NMR values for compound **15** indicate that it has one hydroxyl, two benzoate and three acetate groups. By analogy to similar compounds, one can infer the substitution pattern of the taxane as having a benzoate at C-7, an acetate at C-9, and a hydroxyl at C-l0. Note that the NMR of this compound (see **Tables 1** and **2**) does not show the conformational isomerism that compound **13** does. This structure is confirmed by identification of reaction products.

The acetylation (same conditions as compound **14**) product was identical to compound **12**. In turn, mild hydrolysis of **12** gives **14** as one of its products (see Chapter 6). Benzoylation (cf., compound **18** for conditions) gives compound **16**. Jones' oxidation

was also performed on this compound to confirm the position of the hydroxyl (see compound **20**).

Compound **15** was obtained as a white amorphous powder. IR (KBr, cm^{-1}): 3560, 1735, 1720, 1450, 1365,1270, 1235, 1170, 1110, 1065, 1025, 980, 935, 705. Calculation for elemental analysis for $C_{45}H_{48}O_{14}$ was C: 64.94 and H: 6.23. Found: C: 64.95 and H: 6.47.

The HETCOR spectrum of compound **15** identified an anomalous placement (1.40ppm) for an acetate methyl, similar to that seen in compound **16**. The reason for these acetate methyls coming so far upfield will be discussed later.

Compound 16

Compound **16** was isolated as discussed above. For ^{1}HNMR and ^{13}CNMR values, see Tables 3.1 and 3.2. It is also the benzoylation (cf., compound **18** for conditions) product of compound **15**. It was obtained as a crystalline solid, mp 234-236°C. IR (KBr, cm^{-1}): 3560, 3450, 1735, 1720, 1450, 1365, 1265, 1235, 1225, 1170, 1110, 1065, 1020, 980, 705. Calculation for elemental analysis for $C_{47}H_{50}O_{14} \bullet 2H_2O$ was C: 64.52 and H: 6.22. Found: C: 64.85 and H: 6.14.

The HMBC spectrum of **16** showed correlations between the Ph-CO (δ 165.9) and the *ortho*-protons of the Ph ring (δ 8.04), and the H-2 signal (δ 6.50). The δ 8.04 doublet correlated with the *meta*-Ph-C (δ 129.7) and the *para*-Ph-C (δ 133.5). The Ph-CO signal at δ 165.1 correlated with the H-7 (δ 5.90) and with *o*-Ph-H (δ 8.12), which in turn, coupled with *m*-Ph-C (δ 129.7) and *p*-Ph-C (δ 132.8). In addition, correlation was seen between H-l 8 (δ 2.04) and the C-11 (δ 135.9), C-12 (δ 147.9) and C-13 (δ 78.7).

Similarly, H-19 (δ 1.89) showed long range coupling to the C-3 (δ 44.6), C-7 (δ 70.4) and C-9 (δ 76.5).

Table 3.1. Proton NMR values for compounds: **15**, **16** and **17**.

H-#	Compound **15**	Compound **16**	Compound **17**
2	6.26 (br d, 7.5 Hz)	6.49 (d, 8.0 Hz)	6.51 (d, 7.8 Hz)
3	3.12 (d, 7.5 Hz)	3.21 (d, 8.0 Hz)	3.08 (d, 7.8 Hz)
5	5.00 (d, 7.8 Hz)	5.02 (d, 7.2 Hz)	4.99 (d, 8.4 Hz)
6α	2.64 (m)	2.69 (m)	2.70 (m)
6β	2.05 (m)	2.08 (m)	1.86 (m)
7	5.72 (t, 7.2 Hz)	5.90 (t, 8.4 Hz)	4.60 (t, 8.1 Hz)
9	5.84 (br d, 9.8 Hz)	6.27 (d, 10.5 Hz)	6.68 (d, 11.1 Hz)
10	4.76 (br d, 9.8Hz)	6.73 (d, 10.5Hz)	6.74 (d, 11.1 Hz)
13	5.72 (br t, 7.2 Hz)	5.71 (t, 7.2 Hz)	5.74 (t, 7.8 Hz)
14α	2.30 (m)	2.44 (dd, 14, 7.2Hz)	2.46 (dd, 14, 6.9Hz)
14β	1.90(m)	1.95(m)	2.02(m)
16	1.11 (s)	1.16(s)	1.26(s)
17	1.18(s)	1.18(s)	1.26(s)
18	1.92 (s)	2.04 (s)	1.88 (s)
19	1.83(s)	1.89(s)	1.76(s)
20α	4.49 (d, 7.8 Hz)	4.54 (d, 7.8 Hz)	4.47 (d, 7.8 Hz)
20β	4.17 (d, 7.8 Hz)	4.17 (d, 7.8 Hz)	4.17 (d, 7.8 Hz)
Ac-CH_3	1.40, 2.18, 2.19	1.03, 2.18, 2.22	2.17, 2.22

HMBC showed interaction of the Ac-CO (δ 170.1) with the CH_3 singlet at δ 1.03. This methyl signal is very far upfield for that of an acetate group. The assignment was confirmed by the HETCOR spectrum, which placed the acetate methyl carbon at δ 19.8. The acetate methyl carbon normally appears at about 20ppm.

Table 3.2. Carbon NMR values for compounds: **15**, **16** and **17**.

C-#	Compound **15**	Compound **16**	Compound **17**
1	67.5	75.6	68.1
2	66.2	68.3	68.4
3	44.7	44.6	44.4
4	79.2	79.2	79.5
5	84.6	84.6	84.7
6	34.8	34.9	36.6
7	70.7	70.4	71.5
8	43.5	44.0	44.3
9	79.4	76.5	79.1
10	68.6	68.9	67.8
11	140.0	135.9	136.5
12	142.8	148	147.2
13	79.4	78.7	78.6
14	36.4	36.7	36.9
15	74.4	75.1	74.3
16	27.8	27.8	27.9
17	25.5	25.5	25.6
18	11.3	12.1	11.6
19	12.8	12.8	11.8
20	76.3	74.6	75.9
Ac/Me	22.0, 21.2, 20.4	22.0, 21.2, 19.8	21.0, 22.0
Ac/CO	171.7, 170.9, 169.0	170.6, 170.1, 169.0	169.2, 170.6
Ph/CO	166.0, 165.3	165.9, 165.1, 163.8	165.8, 165.1, 164.9

Compound 17

Compound **17** was identified by NMR (see Tables 3.1 and 3.2) as a novel abeotaxane. The structure was confirmed by acetylation (same conditions as compound **14**) of compound **17** to give taxchinin C (Compound **10**). Benzoylation of compound **17** gave compound **18**. Compound **17** was obtained as a crystalline solid, mp 164-166°C. Specific rotation, $[\alpha]_D$, in CH_2Cl_2 is –77.5°. IR: 3580, 3560, 1735, 1715, 1595, 1575, 1445, 1365, 1310, 1250, 1175, 1105, 1090, 1065, 1020, 985, 930, 910, 860, 840, 705.

Calculation for elemental analysis for $C_{45}H_{48}O_{13}$ was C: 67.83 and H: 6.07. Found: C: 67.62 and H: 6.29.

Compound **17** has three benzoate groups (15 aromatic protons in ^{1}HMNR). The HMBC spectrum showed correlations of all three Ph-CO signals with their corresponding proton signals on the taxane skeleton: δ 165.8 with H-2 (δ 6.51), δ 164.9 with H-9 (δ 6.68) and δ 165.1 with H-10 (δ 6.74). The spectrum also showed a correlation between the H-13 signal (δ 5.74) with the acetate carbonyl at δ 21.0. Interactions were also seen between H-18 and C-11, and C-12 and C-13, as well as that between H-19 and C-3, and C-7 and C-9.

Compound 18

A solution of compound **13** (0.1g) in pyridine (2ml) was cooled and treated with benzoyl chloride (0.3ml). After stirring for 20 h at room temperature, TLC showed reaction to be complete. Water was added to the mixture and the solid filtered out. It was chromatographed with CH_2Cl_2/ligroine on silica to give compound **18**, yield, 0.1g. Compound **18** was also made from compound **17** by benzoylation. ^{1}HNMR (δ) values are: 1.21 (s, 3H, Me); 1.90 (m, 2H, H-6 and H-14); 2.01 (s, 3H, Me); 2.03 (s, 3H, Me); 2.19 (s, 3H, Me); 2.22 (s, 3H, Me); 2.48 (dd (14.1Hz and 7.2Hz), 1H, H-14); 2.68 (br s, 1H, HO-l5); 2.81 (m, 1H, H-6); 3.28 (d (7.5Hz), 1H, H-3); 4.23 (d, 7.5Hz, 1H, H-20); 5.02 (d (7.2Hz), 1H, H-5); 5.74 (t (7.2Hz), 1H, H-13); 5.97 (t (7.8Hz), 1H, H-7); 6.61 (d (7.5Hz), 1H, H-2); 6.64 (d (11.1Hz), 1H, H-9); 6.66 (t (7.5Hz), Ar-H); 6.87 (d (11.1Hz), 1H, H-10); 7.01 (t (7.5Hz), Ar-H); and aryl peaks at 7.12, 7.15, 7.17, 7.19, 7.21, 7.28, 7.31, 7.32, 7.35, 7.48, 7.50, 7.53, 7.55, 7.57, 7.61, 7.63, 7.66, 7.93 (d, 7.5 Hz), 8.06 (d, 7.2Hz). ^{13}C NMR (δ) values are: 13.2, 21.2, 22.0, 22.0, 25.6, 27.8, 35.0, 36.9, 44.2, 44.6,

53.4, 68.2, 68.6, 71.0, 75.8, 78.8, 79.1, 84.7, 127.3, 128.0, 128.2, 128.6, 128.9, 129.1, 129.5, 129.7, 130.0, 130.6, 132.2, 132.6, 132.9, 133.5, 136.2, 147.8, 164.2, 165.3, 165.9, 166.6, 169.0 and 170.7. Calculation for elemental analysis for $C_{52}H_{52}O_{14} \bullet 0.5H_2O$ was C: 68.64 and H: 5.87. Found: C: 68.48 and H: 6.06.

Compound 19

Compound **13** was characterized by way of its acetate (**14**) and its benzoate (**18**). Some doubt however remained over the assignments at the C-9 and C-10 positions. So to determine the placement of the hydroxyl, a Jones' oxidation was performed on compound **13** to give compound **19**. The ^{13}C NMR spectrum of compound **19** showed the signal for a ketone at 191.8ppm. This value is what would be expected for an α,β-unsaturated ketone, placing it at C-10. A ketone at C-9 is generally seen at 207-209ppm. The signal for C-12 also was shifted downfield from approximately δ147-148 to δ157.6, again indicating a C=C-C=O system.

Compound **13** (0.1g) in acetone (3 ml) was treated with Jones' reagent, and the mixture stirred. The progress of the reaction was monitored by TLC. Water was added to the mixture which was then extracted with CH_2Cl_2. The extract was concentrated to dryness and the product chromatographed on silica (CH_2Cl_2-ligroin) to give a crystalline solid (0.06g), which begins to decompose at 240°C.

^{1}HNMR peaks (δ) are: 1.19 (s, 3H, Me), 1.23 (s, 3H, Me), 1.85-2.05 (m, 1H, H-14), 2.15 (s, 3H, Me), 2.15 (s, 6H, 2Me); 2.20 (s, 3H, Me), 2.54 (dd (14.4Hz and 7.2Hz), 1H, H-14), 2.91 (m, 1H, H-6), 3.32 (d (7.2Hz), 1H, H-3), 4.26 (d (7.5Hz), 1H, H-20β), 4.99 (d (5.7Hz), 1H, H-5), 5.49 (dd (8.7Hz and 5.7Hz), 1H, H-7), 5.83 (t (7.5Hz) 1H, H-13), 6.51 (s, 1H, H-9), 6.59 (d (7.5Hz), 1H, H-2), 6.85 (t (7.8Hz), 2Ar-H), 7.27 (t (7.8Hz),

1Ar-H), 7.40 (t (7.2Hz), 2Ar-H), 7.48, 7.51, 7.53, 7.55, 7.62, 7.64, 7.70 (d (7.2Hz), 2Ar-H), 8.05 (d (8.7Hz), 2Ar-H), and 8.08 (d (8.4Hz), 2Ar-H). ^{13}CNMR peaks (δ) are: 14.0, 21.0, 21.9, 25.5, 27.6, 34.7, 37.2,44.1,45.0, 65.9, 69.2, 69.3, 71.2, 74.6, 76.5, 78.7, 79.0, 84.2, 84.9, 127.8, 128.3, 128.7, 128.9, 129.7, 129.8, 129.9, 130.8, 132.8, 132.9, 133.7, 137.6, 157.6, 165.1, 165.9,167.1, 168.9, 170.5, 191.8. Calculation for elemental analysis for $C_{45}H_{46}O_{13}$ was C: 67.96 and H: 5.83. Found: C: 67.63 and H: 5.70.

Compound 20

Compound **15** was also oxidized (same conditions as compound **19**) to give the keto-containing compound **20**. The ^{13}CNMR spectrum showed the C-10 carbonyl signal at 191.2ppm and the C-12 signal at 157.3ppm, indicating an α,β-unsaturated ketone.

^{1}HNMR peaks (δ) are: 1.12 (s, 3H, Me), 1.18 (s, 3H, Me), 1.56 (s, 3H, Me), 2.05 (s, 3H, Me), 2.18 (s, 3H, Me), 2.19 (s, 3H, Me), 2.20 (s, 3H, Me), 2.48 (dd (14.1Hz and 7.2Hz), 1H, H-14), 2.77 (dt (15.9Hz and 8.1Hz), 1H, H-6), 3.23 (d (7.5Hz), 1H, H-3), 4.22 (d (7.5Hz), 1H, H-20), 4.55 (d (7.5Hz), 1H, H-20), 4.99 (d (6.9Hz), 1H, H-5), 5.41 (dd (9.0Hz and 8.4Hz), 1H, H-7), 5.81 (t (7.2Hz) 1H, H-13), 6.19 (s, 1H, H-9), 6.28 (d (7.8Hz), 1H, H-2), 7.44-7.67 (m, Ar-H), 8.03 (d (7.5Hz), 2Ar-H), and 8.16 (d (7.2Hz), 2Ar-H). ^{13}CNMR peaks (δ) are: 13.5, 13.9, 20.0, 21.0, 21.9, 25.5, 27.5, 34.6, 37.0, 44.4, 44.7, 65.9, 68.9, 70.6, 74.4, 76.3, 78.7, 78.9, 84.0, 84.7, 128.3, 128.7, 129.6, 129.8, 130.6, 132.9, 133.7, 137.7, 157.3, 164.6, 165.9, 168.9, 170.5, 170.9, 191.2. Calculation for elemental analysis for $C_{40}H_{44}O_{13} \bullet H_2O$ was C: 63.99 and H: 6.18. Found: C: 63.75 and H: 6.20.

Compound 21

Taxinine J (**21**) crystallized out of a mixture of three compounds dissolved in methanol. The crystals were filtered, washed and recrystallized from methanol to obtain colorless needles, mp 248-249°C (lit. 248-249°C [27]). The filtrate was relatively free of taxinine J, containing only compounds **23** and **24** according to ^{1}HNMR.

Its physical and spectral properties agreed with those given by Woods et al.[71] Carbon NMR values were not readily available and are listed here. ^{13}CNMR peaks (δ): 13.6, 15.8, 20.7, 21.0, 21.3, 21.4, 27.1, 28.3, 31.6, 35.2, 37.6,42.7,47.1, 48.6, 69.8, 70.4, 70.8, 71.6, 75.8, 76.0, 118.3, 118.9, 128.1, 129.0, 130.6, 133.6, 134.1,137.0, 140.3, 146.0, 166.1, 169.2, 169.3, 169.7, 169.8 and 170.6.

Compound 22

Compound **22** is the ozonolysis product of compound **23**, where the exocyclic 4(20)-double bond has been cleaved. It was obtained as a crystalline solid, which begins to decompose at 245°C. IR (KBr, cm^{-1}): 3480, 1755, 1720, 1440, 1370, 1215, 1170, 1095, 1060, 1020, 700. ^{1}HNMR peaks (δ) are: 1.13 (s, 3H, Me); 1.22 (br s, 6H, 2Me); 2.07 (s, 3H, Ac-Me); 2.09 (s, 3H, Ac-Me); 2.14 (s, 3H, Ac-Me); 1.95-2.20 (m, 2H, H-6α and H-6β); 1.95-2.15 (m, 1H, H-14α); 2.48 (dd (14.1Hz and 6.9Hz), 1H, H-14β); 3.69 (d (8.7Hz), 1H, H-3); 4.56 (br s, 1H, H-5); 5.67 (t (7.2Hz) 1H, H-13); 5.79 (dd, 1H, H-7); 6.22 (d (10.2Hz), 1H, H-9); 6.33 (d (7.8Hz), 1H, H-2); 6.72 (d (10.2Hz), 1H, H-10); and 7.42, 7.44, 7.47, 7.52, 7.57, 7.87, 7.90, 7.97 and 7.99 (m, ArH). ^{13}CNMR peaks (δ): 12.1, 13.8, 20.6, 21.2, 21.2, 23.5, 25.5, 27.4, 33.7, 37.6, 37.6, 47.1, 47.6, 65.6, 67.3, 67.8, 68.4, 74.5, 75.3, 75.7, 78.8, 128.4, 128.8, 129.6, 129.7, 132.8, 133.6, 135.9, 137.7, 148.7,

165.7, 169.3 (3×), 170.4, 199.8. Calculation for elemental analysis for $C_{41}H_{46}O_{14}$ was C: 64.55 and H: 6.08. Found: C: 64.17 and H: 6.20.

Compound 23

The mixture of compounds **16** and **23** was chromatographed on a silica column using 1% acetone in benzene as the mobile phase. Compound **23** eluted first in this solvent system. Fractions containing **23** were concentrated and the product was obtained as a crystalline solid, mp 200-203°C. Specific rotation, $[\alpha]_D$, in CH_2Cl_2 is 21.5°. IR (KBr, cm^{-1}): 3540, 1730, 1440, 1360, 1255, 1230, 1170, 1110, 1060, 1020, 705. Calculation for elemental analysis for $C_{42}H_{48}O_{13}$ was C: 66.3 and H: 6.36. Found: C: 66.47 and H: 6.57.

The ^{1}HNMR and ^{13}CNMR (see Table 3.3) spectra of compound **23** showed the presence of four acetate and two benzoate groups. Two singlets at 4.72ppm and 5.15ppm (H-20) and a broad singlet at 5.24ppm (H-5) indicated an exocyclic double bond. A quaternary carbon peak at 69.1ppm (C-1) suggested a 5-7-6-membered ring system rather than a 6-8-6-membered ring system, which would show a peak around 43ppm (C-15). Compound **23** appeared to be an analog of brevifoliol (Compound **24**) with acyloxy substitution at the 2α position.

Table 3.3. Proton and Carbon NMR values for compound **23**.

#	^{1}HNMR	^{13}CNMR
1	-	69.1
2	6.38 (d, 9.0Hz)	68.1
3	3.40 (d, 9.0Hz)	43.3
4	-	139.3
5	5.24 (br s)	75.9
6α	1.86 (m)	34.9
6β	2.05 (m)	-
7	5.56 (dd,10.8, 4.8Hz)	68.8

8	-	45.1
9	6.12 (d, 10.8Hz)	76.2
10	6.73 (d, 10.8Hz)	69.0
11	-	135.5
12	-	148.6
13	5.68 (t, 6.9Hz)	78.9
14α	2.11 (m)	37.9
14β	2.55 (dd, 14.1, 6.9Hz)	-
15	-	75.5
16	1.18 (s)	27.8
17	1.19 (s)	25.8
18	2.10 (s)	12.0
19	1.16 (s)	13.4
20α	5.15 (s)	115.9
20β	4.72 (s)	-
Ac/Me	1.77, 2.08, 2.13, 2.19	20.7, 21.3, 21.2, 21.3
Ac/CO	-	169.5, 169.5, 169.8, 170.6
2-Bz-1"	-	128.9
2-Bz-2",6"	7.95 (d, 7.8Hz)	129.5
2-Bz-3",5"	7.45 (m, 6.3Hz)	128.6
2-Bz-4"	7.57 (m, 6.9Hz)	133.3
2-Bz-CO	-	166.9
10-Bz-1"'	-	130.1
10-Bz-2"',6"'	7.89 (d, 7.5Hz)	129.7
10-Bz-3"',5"'	7.45 (t, 6.3Hz)	128.8
10-Bz-p	7.57 (m, 6.9Hz)	133.4
10-Bz-CO	-	164.2

The general structural assignments were made based on COSY and HETCOR spectra. In order to confirm the location of the benzoate groups, an HMBC spectrum was run. Long range interaction was observed between the *ortho*-protons of one of the benzoates (δ 7.95) and the corresponding carbonyl signal at δ 166.9, which in turn, interacted with the H-2 (δ 6.38). Similar interaction was observed between the *o*-protons (δ 7.89) of the second benzoate and the carbonyl (δ 164.2) and in turn the H-10 (δ 6.73). Thus, compound **23** (2α-benzoyloxy-5,13-diacetyl-brevifoliol) was established as a new member of this group.

Compound 25

Compound **25** was obtained as a crystalline solid, mp 170-171°C (lit. 171-172°C [35]). The physical and spectral properties agreed with those described in the reference.[35] ^{13}CNMR peaks (δ): 13.2, 15.3, 20.8, 21.0, 21.5, 27.2, 27.3, 31.2, 31.9, 34.6, 37.5, 39.4, 40.2, 46.3, 70.0, 70.6, 71.7, 74.8, 76.7, 116.0, 118.4, 128.1, 129.0, 130.6, 134.1, 135.0, 137.2, 145.7, 146.3, 166.1, 169.3, 169.9, 170.2 and 170.7.

Compound 26

Compound **26** was obtained as a colorless crystalline solid, mp 201-203°C (lit. 205-207°C [27]). Its physical and spectral data were identical with those given in the reference.[21]

Compound 27

Compound **27** was obtained as a colorless crystalline solid, mp 197-199°C (lit. 197°C[35]). Its physical and spectral properties agreed with those given in the reference.[21] ^{13}CNMR peaks (δ): 13.8, 15.9, 20.8, 21.0, 21.4, 21.5, 21.5, 27.4, 28.0, 31.6, 34.9, 37.6, 43.0, 47.2, 49.0, 69.9, 69.9, 70.4, 70.7, 71.5, 75.9, 119.1, 133.0, 137.3, 140.1, 169.1, 169.2, 169.4, 169.8, 169.8, and 170.4.

Compounds 28-29

Compound **28** was obtained as a white powder. The spectral data indicated that it was identical with yunnanxane.[13]

Compound **29** was obtained as white powder. Its spectral data showed it to be identical to the taxane reported in the references.[38, 75]

Compound 30

Compound **30** was obtained as a colorless crystalline solid, mp 250-252°C. Its physical and spectral properties were identical with an authentic sample of baccatin VI that was obtained from *Taxus floridana*.[51]

Conclusion

The post-paclitaxel fractions from the reverse-phase column still contained some amount of paclitaxel. Though it might not be economically viable to process these fractions for the remaining paclitaxel, changing some variables in the reverse-phase chromatographic step might increase the overall yield of paclitaxel.

The 11(15→1)-abeotaxanes were isolated in sufficient quantity from the post-paclitaxel fractions to merit further examination. These compounds have many of the structural features known to be of importance in the activity of paclitaxel. The oxetane ring and the benzoate ester at the 2-position are features common to paclitaxel and the abeotaxanes isolated here. The side-chain of paclitaxel is also known to be necessary for its activity. The attachment of a side-chain to one of these abeotaxanes might lead to a new active analog of paclitaxel.

Also of interest in the abeotaxanes were some anomalous ^{1}HNMR values. The values in question were all belonging to acetyl methyl protons. Methyl singlets were identified by HETCOR and HMBC techniques as coming far upfield from the normal position for acetate methyls. The protons of an acetate methyl are deshielded by the electron withdrawing effect of the carbonyl. The normal acetate methyl comes in the 2.0-2.2ppm range.

Methyl signals for compounds **14** (δ1.56), **15** (δ1.40) and **16** (δ1.03) were all identified by the two dimensional techniques mentioned above as belonging to acetate moieties. An explanation for this anomaly lies in the proximity of the acetate methyls to the aromatic rings of the benzoates. Compound **14** has an acetate at the 10-position next to the benzoate at C-9. Compound **15** has an acetate at C-9 next to the benzoate at C-7. Compound **16**, which has the most upfield signal, has an acetate at C-9 flanked by two benzoates at C-7 and C-10.

The methyl of these acetates is spatially constrained above the aromatic ring and becomes shielded by the ring current. In compound **16**, the methyl is sandwiched between two rings and is thus shielded further.

CHAPTER 4
TAXANES FROM *TAXUS* X *MEDIA* HICKSII

Introduction

The bark of *Taxus brevifolia* remains the best natural source of paclitaxel, both in weight percentage of the bio-mass and ease of isolation. Unfortunately, the bark is available only at the expense of the rest of the tree. For sustainable production of paclitaxel, a renewable source of bio-mass must be found. *Taxus* × *media* Hicksii is one of several hybrid cultivars of yew grown in quantity as an ornamental shrub. HPLC analysis has shown the needles to have one of the highest levels of paclitaxel (after *Taxus brevifolia* bark) among the yews. It has been suggested as the new source of paclitaxel.

According to a HPLC study done by Witherup et al, the dry needles of *Taxus* × *media* Hicksii contain about 0.01%, by weight, of paclitaxel. This group also isolated paclitaxel by a method similar to the Poylsciences (Chapter 2) method. The overall yield by this process was 0.006% by weight of the dry needles.[70]

The large-scale isolation procedure, described in Chapter 2, was also performed on the needles of *Taxus* × *media* Hicksii.[51, 55] Chloroform extract solids from the needles were run through a single reverse-phase column, which was eluted with acetonitrile (30-60%) in water to give paclitaxel and five other taxanes in fair amount. A mixture of taxanes, however, co-elutes with paclitaxel, thus limiting its purity.[8, 18] They may be removed by ozonolysis followed by chromatography on a short silica column to give high purity paclitaxel in a yield of 0.012-0.015 % from the dry needles.

10-Deacetylbaccatin III (Compound **2**) is an important precursor to semi-synthetic paclitaxel. Witherup et al. reported that *Taxus* × *media* Hicksii needles contained 0.009% 10-deacetylbaccatin III (according to HPLC analysis).[70] This was not born out in large-scale isolation; no 10-deacetylbaccatin III was isolated. *Taxus* × *media* Hicksii needles may yet become a source for paclitaxel production but it does not have the added benefit of 10-deacetylbaccatin III.

This chapter will deal with the reverse-phase isolation process as applied to the needles of *Taxus* × *media* Hicksii. In addition to the six crystalline compounds, mentioned above, other compounds were isolated upon further processing.

Reverse-Phase Chromatography

Isolation of paclitaxel and other taxanes was carried out on a pilot-plant scale from the needles of *Taxus* × *media* Hicksii (200 lbs. of the dried material), which were donated by Hauser Inc., Boulder CO. Processing proceeds like that for *Taxus brevifolia* bark: extraction of the dried needles in methanol, concentration of the extract, partitioning between water and chloroform, and concentration of the chloroform layer to dryness (5% by weight of needles). Each extraction step is monitored by UV absorbance at 275nm to insure the efficiency of extraction.

The chloroform extract contains a large amount of lipid soluble compounds (e.g., waxes, chlorophylls and steroids). There was some concern that this material might tend to clog the reverse-phase column causing the pressure to rise. This was not the case. Sample preparation allows the C-18 bonded silica gel to take up these non-polar components. The stationary phase appears to dissolve the waxy material that is insoluble in the mobile phase.

The $CHCl_3$ extract was first dissolved in acetonitrile to which equilibrated packing material (C-18 bonded silica) was added. As this is stirred, water is slowly added until a concentration of 25% acetonitrile is reached. The mixture is allowed to settle and then the supernatant is poured off. The solid material is then placed on top of the column and the supernatant is pumped onto the column using a diaphragm pump. The column is then eluted using increasing amounts of acetonitrile and water.

Two such columns were run: 2.5kg of the extract (from 50kg of the dry needles) was loaded on a 6"×6' column and 600g (from 12kg of needles) on the 4"×4' column. The columns were then developed with a step gradient: 30-60% (in increments of 5%), during which, most of the taxanes of interest were eluted. One more taxane eluted with methanol. After the elution of the taxanes is complete, the column can be completely stripped of these lipophilic components (ligroine/ethyl acetate/methanol), then re-equilibrated with the acetonitrile/water and made ready for reuse.

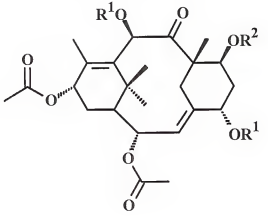
Isolation of Taxanes

After the sample was loaded, elution with 30-35% acetonitrile/water gave large amounts of water-soluble, polar constituents, which accounted for the bulk of the UV absorbance at 275nm. TLC showed a strong, fast-moving spot that turned red upon charring with sulfuric acid. It was present in most of the early fractions and continued into the fractions containing brevifoliol (Compound **24**). The compound was not a taxane, but was identified as *para*-hydroxybenzaldehyde. This is a commonly occurring compound in plants.[55]

Elution with 35-40% solvent mixture gave a number of minor components, followed by a major component, identified as brevifoliol. This elutes slightly after 10-deacetyl-

baccatin III in reverse-phase chromatography (and consequently HPLC). Witherup's study was done before the discovery of brevifoliol and so may have mistaken one compound for the other. This is one of the pitfalls of relying solely on HPLC for characterization of a complex (and as yet unknown) mixture of compounds.[70]

Fractions containing brevifoliol (Compound **24**) as the major component were combined and concentrated until solids began to appear. After 2-3 days, the solid was filtered and the filtrate was concentrated to a syrup. A portion of this syrup (15g) was chromatographed on silica gel (150g) using the solvent sequence: CH_2Cl_2, 2-5% acetone in CH_2Cl_2, 2-5% methanol in CH_2Cl_2 and 10% methanol in CH_2Cl_2.



	R^1	R^2
31	H	Ac
32	Ac	Ac
33	H	H

Figure 4.1. 2(3→20) abeotaxanes

The 2-5% acetone in CH_2Cl_2 eluate (4g) was re-chromatographed on silica gel to give two crystalline compounds that were identified as *p*-hydroxybenzaldehyde (eluting with 2% acetone, 0.4g) and apigenin (5% acetone, 0.1g). Apigenin, 4',5,7-trihydroxy-flavone, is also a commonly occurring compound in plants. The mother liquors from those fractions having *p*-hydroxybenzaldehyde were chromatographed on Florisil with the same solvent sequence. Florisil is a magnesium silicate gel, which traps flavonoids

and other phenolic compounds by chelation. This was followed by preparative TLC (CH_2Cl_2/acetone/methanol, 26:3:1) which gave two taxanes belonging to the 2(3→20) abeotaxane group (compounds **31** (0.12g) and **33** (0.08g), Figure 4.1), compound **34** (0.12g, Figure 4.3) and taxinine M (Compound **36**, 0.06g, Figure 4.2). A portion of compound **31** was acetylated to give compound **32** (Figure 4.1).

The 2-5% methanol in CH_2Cl_2 eluate (3g) gave brevifoliol as the major product. The fractions collected with 5% methanol in CH_2Cl_2 (3g) on further fractionation gave 10-deacetylpaclitaxel (Compound **8**, 0.2g, Figure 2.2), and finally a mixture (0.3g) of 10-deacetylpaclitaxel-7-xyloside (Compound **4**) and 10-deacetylpaclitaxel-C-7-xyloside (Compound **5**).

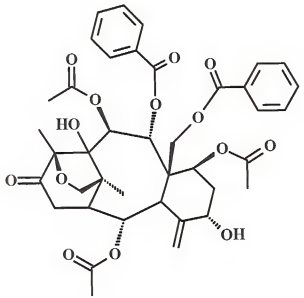
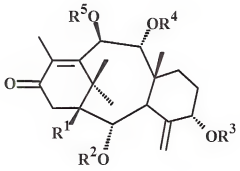


Figure 4.2. Taxinine M

Elution with 45-50% acetonitrile/water started the elution of compounds **37** and **38**, followed by paclitaxel (**1**), all of which crystallized together from the fractions, as they stood for 2-8 days. The crude crystals (25g from 12kg needles) were processed by two

methods. In the first, a solution in $CHCl_3$/ligroine (3:1, 250ml) was chromatographed on silica (150g), with the eluent changed to $CHCl_3$, 2% acetone, 5% acetone, 2% methanol and 5% methanol, all in $CHCl_3$. The mixture of compounds **37** and **38** (Figure 4.3) appeared first (2-5% acetone), followed by paclitaxel (2% methanol). Concentration of the appropriate fractions gave the mixture of **37** and **38** (12 g, 0.1%). A second method of processing was used on material obtained from 2.5kg of the extract (from 50kg of needles) run on a 6"-diameter column. The crude mixture of paclitaxel and compounds **37** and **38** (95g) was processed by ozonization (without the intermediate silica column) in 30g portions. The product chromatographed on silica as before. By this method, the yield of pure paclitaxel was 7.5g (0.015%).



	R^1	R^2	R^3	R^4	R^5
34	OH	Ac	H	Ac	Ac
35	OH	Ac	Ac	Ac	Ac
37	OH	H	Cinn.	H	Ac
38	OH	H	Cinn.	Ac	H
39	OH	Ac	Cinn.	Ac	Ac
40	H	Ac	Cinn.	Ac	Ac

Figure 4.3. 13-Oxo-taxadienes

Elution with 55-60% acetonitrile/water gave another crystalline taxane. The crude crystals were filtered and recrystallized from acetone/hexane to give compound **39** (Figure 4.3) as colorless needles.

Elution with methanol deposited crystals of compound **40** (Figure 4.3). This compound was recrystallized from acetone/ligroin. Further elution with methanol

produced large amounts of crystals consisting of sitosterols (commonly occurring plant steroids).

The column was then washed with ethyl acetate, ligroin and methanol mixtures and re-equilibrated with 25% acetonitrile/water to ready it for its next use.

Characterization of Compounds

p-Hydroxybenzaldehyde

p-Hydroxybenzaldehyde was identified by ^{1}HNMR and ^{13}CNMR. Its spectral data was found to be identical with an authentic sample. In addition, the 2,4-dinitrophenyl-hydrazone derivative was formed for the plant isolate and the authentic sample. Both were identical.

Apigenin

Apigenin was identified first on TLC. Ferric chloride solution sprayed on the TLC plate will show a red spot in the presence of phenolic compounds. The suspected flavonoid showed a characteristic UV spectrum (maxima: 210, 265 and 330nm). Also characteristic was a shift upon adding base (KOH; maxima: 220, 275 and 390nm). NMR spectra, before and after acetylation to determine the number of hydroxyls, identified the flavonoid as apigenin.

Brevifoliol

Fractions from 40% acetonitrile/water were partially concentrated. The solid that separated was filtered, and decolorized by dissolving it in CH_2Cl_2 and passing it through a column of Florisil (3g per gram of the sample). Brevifoliol (Compound **24**) crystallized from acetone/ligroine (2:1), yield 0.02%. Melting point: 202-204°C (lit. 200-203°C [3]).

Specific rotation $[\alpha]_D$ is –27°. Calculation for elemental analysis for $C_{31}H_{40}O_9$ was C: 66.89 and H: 7.24. Found: C: 67.13 and H: 7.35.

Compound 31

The ^{13}C spectrum of compound **31** showed four peaks in the alkene carbon region: 124.8, 133.9, 135.3 and 138.6. An attached proton test (APT) showed that the peak at 124.98ppm carried one H, while the others were quaternary. Thus, the presence of only one vinylic proton suggested that compound **31** had a rearranged 6-10-6-taxane skeleton (rearranged B-ring). This is a 2(3→20) abeotaxane, such as that in taxine A.[2, 27]

2α,7β,13α-Triacetoxy-5α,10β-dihydroxy-9-keto-2(3→20)abeotaxane (Compound **31**) was later isolated from the stems of *Taxus cuspidata* as an amorphous solid and spectral data agree with that here.[31] Crystalline solid (acetone/hexane), yield, 0.12 g (0.005% of the dried needles), mp. 172-174°C. $[\alpha]_D$ is –147°. ^{1}HNMR ($CDCl_3$) δ: 1.18 (s, 3H), 1.20 (s, 3H), 1.31 (s, 3H), 1.65 (d (8Hz), 1H, H-3), 1.94 (s, 3H), 1.96 (m, 1H, H-14), 2.02 (s, 6H), 2.08 (m, 2H, H-6α/β), 2.19 (s, 3H), 2.70 (m, 2H, H-3 and H-14), 4.21 (s, 1H, OH, D_2O exchangeable), 4.49 (br s, 1H, H-5), 5.06 (dd (11.5Hz and 4.5Hz), 1H, H-7), 5.35 (d (9.5Hz), 1H, H-13), 5.46 (s, 1H, H-10), 5.65 (d (9.75Hz), 1H, H-20) and 5.71 (dd (9.75Hz and 1.5Hz), 1H, H-2). ^{13}CNMR: 46.8 (C-1), 70.3 (C-2), 34.8 (C-3), 138.3 (C-4), 68.2 (C-5), 35.4 (C-6), 70.5 (C-7), 52.5 (C-8), 213.2 (C-9), 76.7 (C-10), 134.0 and 135.3 (C-11 and C-12), 69.7 (C-13), 26.3 (C-14), 37.1 (C-15), 35.1 (C-16), 23.8 (C-17), 18.2 (C-18), 20.7 (C-19), 124.8 (C-20), [20.8, 20.9, 21.2 (CH_3CO)], [170.0, 170.1, 170.1 (CH_3CO)]. FAB-MS: m/z 515 $[M+Na]^+$, 475 $[M+1-18]^+$, 433 $[M+1-60]^+$, 415 (475–60), 373 (433–60), 313 (373–60), 295 (373–18–60), 267 (295–28).

Calculation for elemental analysis for $C_{26}H_{36}O_9$ was C: 63.40 and H: 7.37. Found: C: 63.11 and H: 7.52.

Compound 32

Acetylation of compound **31** (50mg, Ac_2O (2ml), pyridine (0.5ml) at 80°, 3hr) gave compound **32**. It was crystallized from acetone/ligroine, 35mg, mp. 240-241°. ^{1}HNMR (δ): 1.11 (s, 3H), 1.26 (s, 3H), 1.29 (s, 3H), 1.70-2.20 (m, 5H), 1.95 (s, 3H), 2.00 (s, 3H), 2.06 (s, 3H), 2.14 (s, 3H), 2.17 (s, 3H), 2.24 (s, 3H), 2.72 (m, 2H), 5.22 (dd (12Hz and 3Hz), 1H), 5.4-5.51 (m, 3H), 5.72 (dd (10Hz and 2Hz), 1H) and 6.30 (s, 1H). ^{13}CNMR: 46.8 (C-1), 70.4 (C-2), 32.3 (C-3), 138.8 (C-4), 69.3 (C-5), 35.4 (C-6), 70.8 (C-7), 53.1 (C-8), 205.6 (C-9), 77.8 (C-10), 133.1 (C-11), 128.6 (C-12), 70.0 (C-13), 27.1 (C-14), 37.8 (C-15), 31.6 (C-16), 25.0 (C-17), 16.8 (C-18), 20.3 (C-19), 123.7 (C-20), [20.7, 20.7, 21.3, 21.4, 21.4 (CH_3CO)], and [169.4, 169.7, 170.1, 170.3, 170.3 (CH_3CO)]. FAB-MS: m/z 599 $[M+Na]^+$, 577 $[M+H]^+$, 457, 415, 397, 373, 355, 313, 295 and 253. Calculation for elemental analysis for $C_{30}H_{40}O_{11}$ was C: 62.49 and H: 6.99. Found: C: 62.78 and H: 7.12.

Compound 33

The ^{1}HNMR and ^{13}CNMR spectra for compound **33**, 2α,13α-diacetoxy-5α,7β,10β-trihydroxy-9-keto-2(3→20)abeotaxane, showed close relationship to compound **31**, but having one less acetate moiety. Acetylation of **33** also gave **32**, proving that the ring system and pattern of oxygenation were the same for **31** and **33**. Compound **33** had been reported as deaminoacyl taxine A (from *Taxus baccata* needles) by Appendino et al.[2] Its ^{1}HNMR and ^{13}CNMR spectra were in good agreement with those reported, except for

two carbon signals: 38.9 (C-6) and 47.0 (C-1), as opposed to 30.9 and 44.8, respectively given in the reference.[2] A new sample of compound **33** was isolated and it too showed the same signals: 38.9 and 47.0. Furthermore, the signal at 47.0 (C-1) is in line with that seen in compounds **31** and **32** and with those in the literature for taxine A and other similar compounds.[2,27] For the signal at 38.9 (C-6), no example with hydroxyls at both C-5 and C-7, other than the deaminoacyl taxine A is known and those in which one or both are acetylated appear near 35ppm. It is known that a hydroxy substituent deshields the β-carbon to a greater extent (~3ppm) than an acyloxy substituent.[61] The presently observed peak at 38.9ppm (versus 30.8) appears justifiable based on deshielding. Despite these differences, compound **33** is considered to be the same as the deaminoacyl taxine A.[2]

Compounds 34-35

Compound **34** was purified by preparative TLC and obtained as a powder, yield 0.005% of the needles. The ^{1}HNMR and ^{13}CNMR spectra for compound **34**, triacetyl-5-decinnamoyl taxicin I, showed that it was identical to the compound obtained from the needles of *Taxus baccata* by Barboni et al.[4]

Acetylation of compound **34** gave the mono-acetylation product (Compound **35**), which has not previously been described. Spectral data for compound **35** follows. ^{1}HNMR: 0.93 (s, 3H), 1.21 (s, 3H), 1.69 (s, 3H), 1.72-1.83 (m, 2H, H-6α/β), 1.98 (s, 3H), 2.07 (s, 3H), 2.08 (m, 1H, H-7), 2.09, (s, 3H), 2.16 (s, 3H), 2.15 to 2.18 (m, 1H, H-7), 2.25 (s, 3H), 2.62 (d (20Hz), 1H, H-14), 2.78 (d (20Hz), 1H, H-14), 3.38 (d (7Hz), 1H, H-3), 4.70 (s, 1H, H-20), 5.24 (br s, 1H, H-5; in COSY spectrum, this was coupled to H-6α/β at 1.72 and 1.83), 5.34 (s, 1H, H-20), 5.59 (d (7Hz), 1H, H-2), 5.92 (d (10Hz), 1H,

H-9) and 6.10 (d (10Hz), 1H, H-10). ^{13}CNMR: 13.7, 17.4, 19.8, 20.7, 20.9, 21.1, 21.3, 27.5, 28.5, 34.3, 42.7, 43.6, 44.7, 45.8, 71.9, 72.9, 75.2, 77.7, 117.3, 113.8, 141.9, 151.9, 169.6, 169.9, 170.1, 171.8 and 198.8. HRFAB-MS: $[M+1]^+$, 535.2966, Calculated mass for $C_{28}H_{39}O_{10}$: 535.3002.

Compound 36

Taxinine M (Compound **36**) was first isolated from the bark of *Taxus brevifolia* by Beutler et al.[7] It was isolated here as an amorphous solid (yield, 0.003% of the needles) and identified by comparison of the ^{1}HNMR and ^{13}CNMR spectral data.

10-Deacetylpaclitaxel

10-Deacetylpaclitaxel has the same R_f on TLC as that of brevifoliol, but differing in its color tests (with sulfuric acid spray and charring on a hot plate). 10-Deacetylpaclitaxel shows a light-brown color and brevifoliol shows a dark-blue spot. Brevifoliol containing fractions were chromatographed on silica. 10-Deacetylpaclitaxel was obtained from the 2-5% methanol/CH_2Cl_2 eluate and purified further by preparative TLC (7% methanol/ CH_2Cl_2) and crystallized from acetonitrile to give needles: Yield, 0.2g (0.008% of the needles), mp. 194-196°C. The compound was identified by comparison of HPLC and NMR data with that of an authentic sample.

Xylosides

Brevifoliol containing fractions were chromatographed on silica. Crystals formed in fractions that eluted with 5-10% methanol/CH_2Cl_2. A ^{1}HNMR spectrum of the crystals showed the presence of two xylosidic taxanes. HPLC analysis indicated that the two taxanes were 10-deacetylpaclitaxel-7-xyloside (Compound **4**) and 10-deacetylpaclitaxel-

C-7-xyloside (Compound **5**). The mixture (0.3g) was applied to a column of C-18-bonded reverse-phase silica gel (25g, 15-35μm) and the column developed with 35% acetonitrile/water. Based on the results of analytical HPLC, fractions containing the two components were combined separately and concentrated to dryness. Each compound was identified by comparison of spectral data to that of authentic samples. The major component, 10-deacetylpaclitaxel-C-7-xyloside, was crystallized from acetone to give needles, mp. 247-249°C, yield 120mg (0.004%). The slower, minor component, 10-deacetylpaclitaxel-7-xyloside, was likewise crystallized to give 70mg (0.002%), mp. 218-220°C.

Compounds 37-38

A portion (1g) of the mixture of compounds **37** and **38** was applied to a C-18 reverse-phase column (25g) in 40% acetonitrile/water, and eluted with 45 and 50% acetonitrile/water. After one week, the fractions with the crystals were filtered into groups. Although compounds **37** and **38** were separated, such that each contained the other to the extent of 10% or less, further recrystallizations gave worse mixtures suggesting that isomerization was taking place in solution. The ^{1}HNMR and ^{13}CNMR spectra of the crystalline compounds **37** and **38** gave evidence for mixtures of the two compounds. From the spectral data, these two were identified as 5-cinnamoyl-9-acetyltaxicin I (Compound **37**) and 5-cinnamoyl-10-acetyltaxicin I (Compound **38**), as described by Chmurney et al. from *Taxus* × *media* Hicksii, and by Appendino et al. from the needles of *Taxus baccata*.[2, 18] The mixture of **37** and **38** on acetylation gave a single compound, mp. 238-241°C. The NMR spectra showed it be identical with compound **39**.

Paclitaxel

Two batches of $CHCl_3$ extract were processed. Crude crystals, from the reverse-phase fractions, were a mixture of paclitaxel and compounds **37** and **38**. Silica chromatography was used to separate the compounds giving paclitaxel (95% purity) with a yield of 0.012%. The crude mixture of paclitaxel and compounds **37** and **38** processed directly by ozonization gave pure paclitaxel with a yield of 0.015%. Its spectral and physical data were the same as those of an authentic sample.

Compound 39

Crude crystals of compound **39** were filtered and recrystallized from acetone/hexane to give colorless needles, yield 0.02%. The spectral data showed that it is the 5-cinnamoyl-2,9,10-triacetylacetyltaxicin I (Appendino et al.[2] and Baxter et al.[6]). Physical data: mp. 238-241°C, specific rotation $[\alpha]_D$ ($CHCl_3$): 214° (lit.[6] 218°), FAB-MS: m/z 645 $[M+Na]^+$, 623 $[M+H]^+$, 475 $[(MH^+)$-148 (cinnamoyl)], 415 (475–AcOH), 355 (415–AcOH), 295 (355–AcOH). Calculation for elemental analysis for $C_{35}H_{42}O_{10} \cdot H_2O$ was C: 65.61 and H: 6.92. Found: C: 66.00 and H: 6.72.

Compound 40

This compound was recrystallized from acetone/ligroine, yield 0.02%. The NMR spectral data identified it as 5-cinnamoyl-2,9,10-triacetyltaxicin II.[2,6] Physical data: mp. 265-267°C, specific rotation $[\alpha]_D$ ($CHCl_3$): 133° (lit. [15] 137°), FAB-MS: m/z 607 $[M+H]^+$, 459 $[(MH^+)$–148 (cinnamoyl)], 399 (459–AcOH), 339 (399–AcOH), 279 (339–AcOH). Calculation for elemental analysis for $C_{35}H_{42}O_9$ was C: 69.02 and H: 7.03. Found: C: 69.29 and H: 6.98.

Conclusion

The needles of the ornamental hybrid *Taxus* × *media* Hicksii or another cultivar may one-day be the main source of paclitaxel. *Taxus* × *media* Hicksii needles are a renewable resource and do contain sufficient paclitaxel to serve as that source.

Application of the reverse-phase process (Chapter 2) developed for the bark met with some success in its use on the needles. The yield of paclitaxel from this process was as high as 0.015% (twice that obtained with standard-phase processing, 0.006%). Additionally, the isolation of 10-deacetylpaclitaxel (0.008%) and the two xylosides increase the potential yield from this source.

The process was not as efficient as that for the bark because of the co-eluting compounds present in the needles. An additional step is necessary to produce pure paclitaxel. HPLC analysis had indicated the presence of 10-deacetylbaccatin III in the needles but that was not the case. The lack of this semi-synthetic precursor to paclitaxel limits the value of *Taxus* × *media* Hicksii needles.

CHAPTER 5
TAXUS BREVIFOLIA HEARTWOOD

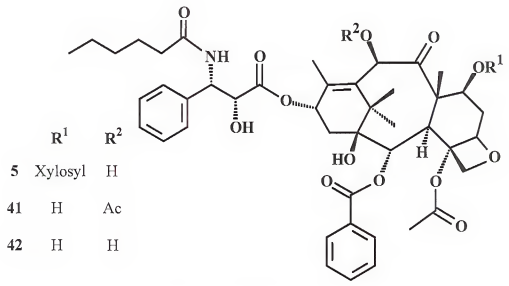
Introduction

The bark of the pacific yew tree has received a great deal of attention. This is not surprising since it has been the major source of paclitaxel. What is surprising is that very little work has been published on the phytochemistry of its heartwood. Thousands of tons of bark have been harvested to supply paclitaxel for clinical use. The trees that were harvested for our studies were in the range of four to eight inches in diameter. The bark of the yew tree is rather thin (about one-eighth of an inch). A quick calculation will show that the bark comprises about 10% of the volume of the average tree.

In our study, we contracted to purchase ten thousand pounds of bark. We also received shipment of a few hundred pounds of heartwood. Somewhere in the order of one hundred thousand pounds of wood met its fate as compost, sawdust or firewood. The tree is too small and slow growing to even be of use as lumber. The question remains does the wood contain sufficient amounts of paclitaxel or other taxanes to merit large-scale processing?

Vidensek et al. did an HPLC study of various parts of *Taxus brevifolia* to determine levels of paclitaxel.[66] They found an average amount of paclitaxel in the heartwood of 0.0006% (0.015% in the bark). According to these numbers, the large scale processing of the wood might not be worthwhile due to the sheer volume of material to process. This study however made no attempt in determining quantities of other taxanes.

The reverse-phase isolation process, described in Chapter 2, was applied to the heartwood of *Taxus brevifolia* to determine its constituents. The processing of the material via reverse-phase and standard-phase chromatography to obtain pure products has born out that paclitaxel (Compound **1**, Figure 1.1) is not present in the wood, but rather paclitaxel C (Compound **41**, Figure 5.1). 10-deacetylpaclitaxel C (Compound **42**, Figure 5.2) and 10-deacetylpaclitaxel C-7-xyloside (Compound **5**, Figure 2.2) are also present in the wood along with many other taxanes.



	R^1	R^2
5	Xylosyl	H
41	H	Ac
42	H	H

Figure 5.1. Paclitaxel C Analogs

Reverse-Phase Processing

One drum full of wood (65 pounds) was extracted with methanol. The extract was concentrated by vacuum distillation. Partitioning of the extract between water and chloroform, and concentration of the chloroform layer to dryness gave 650g of solid material.

The chloroform extract solids (500g) were prepared for large-scale reverse-phase column chromatography (4"×4' column). The $CHCl_3$ extract was first dissolved in 1.5 liters of acetonitrile to which equilibrated (25% acetonitrile/water) packing material was added. As this is stirred, water is slowly added until a concentration of 25% acetonitrile is reached (6 liters overall). The mixture was allowed to settle and the supernatant poured off. The solid material poured onto the top of the column and the supernatant pumped onto the column using a diaphragm pump, which can handle a suspension without clogging.

The column was eluted using a step-gradient of acetonitrile/water. The fractions were monitored with TLC and the solvent-system changed accordingly. HPLC of the fractions did not give useful information. Elution began with 25% acetonitrile/water. Following steps were 35%, 40%, 50% acetonitrile/water and then 50% acetonitrile/10% methanol/water. The column was then washed with 25% ethyl acetate/methanol, followed by 25% ethyl acetate/25% ligroin/ methanol.

Several of the column fractions deposited crystalline products on standing for a few (2-5) days. These, as well as the fractions that did not crystallize, were investigated. Filtering these solids and recrystallization gave the pure compounds in a few cases. Chromatography on a silica column was used to purify other components of the mixtures.

Isolation of Taxanes and Other Compounds

Fractions eluting with 35-40% acetonitrile/water formed the greatest amount of crystals. The crystals were recrystallized from acetone with a few drops of water. The crystals were identified by ^{1}HNMR and ^{13}CNMR to be baccatin IV (Compound **43**, Figure 5.3).[22] The mother liquors were run on a silica column starting with CH_2Cl_2 and

increasing amounts of acetone up to 10%. The earliest compound to elute was a non-taxane compound. It was identified on TLC by the light blue color it produced when developed with sulfuric acid spray and heating on a hotplate. It was identified mainly by ^{1}HNMR and ^{13}CNMR as a lignan (Tsugacetal, Compound **44**, Figure 5.2) which had been isolated from the Taiwan hemlock (*Tsuga chinensis*) tree.[23] The next compound to elute was baccatin IV, which was recrystallized from ether. The filtrate then produced crystals of a second compound. This compound was identified by NMR as 1β-hydroxybaccatin I (Compound **45**, Figure 5.4). Compound **46** (Figure 5.3) eluted with 10% acetone. It was crystallized from ether and identified by NMR as 13-acetyl-7,9-dideacetylbaccatin III.

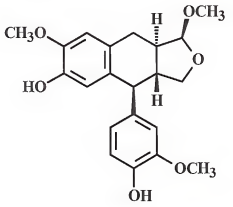
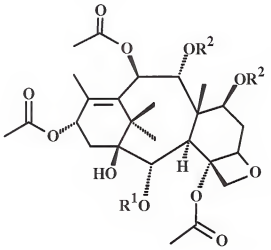


Figure 5.2. (+)-Tsugacetal

Fractions eluting with 40-45% acetonitrile/water gave crystals, which were recrystallized with acetone and filtered to give 10-deacetylpaclitaxel C-7-xyloside (Compound **5**). Further crystallization from the filtrate with acetone and a few drops of water gave baccatin IV (Compound **43**).

The eluates from 45% acetonitrile/water gave a crystalline material that was filtered and recrystallized twice from acetone using charcoal. The product, a colorless crystalline

solid, was identified as 10-deacetylpaclitaxel-C-7-xyloside by analytical and spectral data.[59] The overall yield of the xyloside was greater than 0.01%. The filtrates from the crystallization of the above xyloside were concentrated and applied to a column of silica gel in chloroform. The elution sequence was chloroform, 2% acetone, 5% acetone, 2% methanol, 5% methanol and 10% methanol in chloroform.



	R^1	R^2
30	Bz	Ac
43	Ac	Ac
46	Bz	H

Figure 5.3. Oxetane Compounds

Fractions from the silica column eluting with 2% acetone in chloroform showed two major spots on TLC (developed in 10% acetone/CH_2Cl_2). Running the TLC in 50% ethyl acetate/ligroine showed greater separation of four or five major components. The fractions were placed on a silica column and eluted with 20-40% ethyl acetate/ligroine. The first compound to elute (at 20% ethyl acetate) was identified as 5,13-dideacetyl-taxusin (Compound **47**, Figure5.5). Compound **27** (Figure 3.4) eluted with 25% ethyl acetate/ligroine. 30% ethyl acetate/ligroine eluted two compounds. Baccatin I

(Compound **48**, Figure 5.4) eluted first, then a mixture of the two and finally baccatin VI (Compound **30**, Figure 5.3, see also Chapter 3).

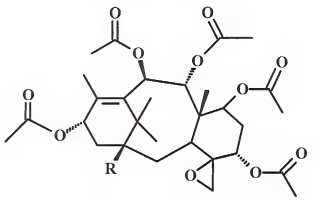


Figure 5.4. Epoxide Compounds

Fractions from the silica column (run on the mother liquors from 45% acetonitrile/ water fractions) eluting with 2% methanol in chloroform was isolated and purified by crystallization from aqueous acetonitrile. The product (yield 0.006%) was the major component of the filtrate and was identified as 10-deacetylpaclitaxel C (Compound **42**).

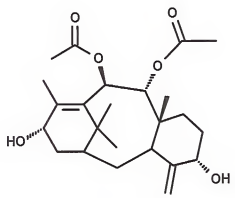
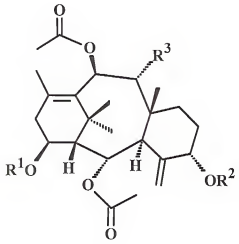


Figure 5.5. 5,13-Dideacetyltaxusin (Compound **47**)

The eluates from 50% acetonitrile/water showed a fast-moving spot on TLC. Fractions were run on a silica column. Fractions from the silica column were tested in

cell culture assay on L1210 cells. Later fractions showed activity comparable to paclitaxel. Compound **49** (Figure 5.6) eluted with 10-20% ligroine in CH_2Cl_2. It was identified as a yunnanxane-like compound with oxygenation at the C-14 position. Elution with 2-5% acetone in CH_2Cl_2 gave compound **26** (Figure 3.4). Fractions eluting with 10% acetone gave paclitaxel C (Compound **41**). This was the compound responsible for the activity in the L1210 assay.



	R^1	R^2	R^3
29	αMeBu	Ac	H
49	Ac	Ac	OAc
50	αMeBu	Ac	OAc
51	αMeBu	H	H

Figure 5.6. Yunnanxane Analogs

Early fractions eluting from the reverse-phase column with 25% ethyl acetate in methanol gave a crystalline material. The crystals were recrystallized from ligroine and identified as phenanthrene. Fractions coming just after those containing phenanthrene were examined. 10g of the concentrated fractions (viscous liquid) were dissolved in ligroine and applied to a silica (50g) column. Elution proceeded with increasing amounts of ethyl acetate and methanol. Fractions eluting with 10% ethyl acetate/ligroine were concentrated. A few small crystals formed. Dissolution of the concentrate in methanol gave a greater degree of crystallization. The compound proved to be stearic acid.

Fractions eluting with 30% ethyl acetate/ligroine gave a mixture of compounds. Fractions eluting with 30% ethyl acetate and 2% methanol in ligroine gave a crystalline compound (Compound **50**, Figure 5.6) with oxygenation at the C-14 position.

The mixture of compounds (1.45g) that eluted with 30% ethyl acetate/ligroine was placed on a silica (15g) column. The column was eluted with increasing CH_2Cl_2 in ligroine. Elution with 20% CH_2Cl_2 in ligroine gave 200mg of compound **29**, which was also obtained from the bark. Increasing the percent CH_2Cl_2 in ligroine from 20 to 100 in 10% intervals eluted a mixture of compounds **29** and **50** (Figure 5.6). Elution with 0-2% acetone in CH_2Cl_2 gave compound **51** (Figure 5.6), which also has oxygenation at the C-14 position.

Characterization of Compounds

The compounds isolated from the heartwood of *Taxus brevifolia* were identified mainly by NMR spectra. Several taxanes isolated from the wood were previously isolated in this lab from the bark (Chapters 2 and 3). These compounds were identified by comparison with the previously obtained samples.

Baccatin IV

Baccatin IV (Compound **43**) was the most abundant taxane in the wood. Not all of the fractions containing it were worked with. More than 7 grams of pure crystalline baccatin IV were isolated (yield 0.031%). The spectral properties were identical with those in the literature.[22]

Compound 44

Tsugacetal (Compound **44**) was identified mainly by ^{13}CNMR. It is a lignan that was isolated from the Taiwan hemlock tree. When first recrystallized from ether/ligroin, the compound is colorless. It becomes brown with age.[23]

Compound 46

Compound **46** was crystallized from ether (0.5g, yield 0.002%) and identified by NMR as 13-acetyl-7,9-dideacetylbaccatin III. This compound was isolated previously from the needles of *Taxus canadensis*.[29, 74] It was used to make the 9-dihydro analog of paclitaxel, which exhibited cytotoxicity close to that of paclitaxel and 100 times the water solubility.[36]

Compound 47

5,13-dideacetyl-taxusin (Compound **47**) was isolated as an amorphous solid in low yield. Its ^{1}HNMR values agree with that reported by Della Casa de Marcano et al.[21]

Paclitaxel Analogs

10-Deacetylpaclitaxel C-7-xyloside (Compound **5**) crystallized from the reverse-phase column fractions. It was recrystallized from acetone to give 2.9g from 50 lbs. of wood (yield 0.013%). The compound was identical to that isolated from the bark (see Chapter 2).

10-Deacetylpaclitaxel C, mp. 168-70°C, $[a]_D$ –45.6° (c = 1%, chloroform). ^{1}HNMR spectral data, ($CDCl_3$): 0.84 (t, 7Hz, CH_3 of the hexanoyl), 1.125 (s, CH_3-16), 1.25 (s, CH_3-17), 1.56 (t, 7.2Hz, CH_2 of the hexanoyl), 1.74 (s, CH_3-19), 1.81 (s, CH_3-18), 1.82 (m, H-14β), 1.95 (m, 6-H), 2.25 (m, 6-H), 2.25 (m, H-14α), 2.34 (s, Ac-4), 3.72 (d,

5.1Hz, 2'-OH), 3.88 (d, 7.2Hz, H-3), 4.27 (m, H-7), 4.2 (H-20), 4.3 (H-20), 4.68 (dd 2.5Hz and 5.1Hz, H-2'), 4.92 (d, 7.8Hz, H-5), 5.21 (s, 10-H), 5.57 (dd, 9.3Hz and 2.5 Hz, H-3'), 5.68 (d, 6.9Hz, H-2), 6.18 (t, 9Hz, H-13), 6.38 (d, 9.3Hz, NH), and 7.5-8.11 (H-aromatic). ^{13}CNMR spectral data: (δ) 9.9, 13.9, 14.3, 20.7, 22.3, 22.5, 25.4, 26.5, 31.3, 35.8, 36.6, 36.9, 43.1, 46.4, 54.4, 57.6, 72.0, 73.1, 74.5, 74.8, 78.6, 81.1, 84.1, 126.9, 128.2, 128.7, 128.9, 129.2, 130.2, 133.7, 136.1, 138.1, 138.2, 166.9, 170.3, 172.8, 173.1, and 211. Calculation for elemental analysis for $C_{44}H_{55}NO_{13}$ was C: 65.57, H: 6.88 and N: 1.74. Found: C: 65.65, H: 6.78 and N: 1.75.

Paclitaxel C was isolated as an amorphous solid in very low yield (32mg). The ^{1}HNMR and ^{13}CNMR values were in agreement with those listed by Barboni et al. who isolated it from the roots of *Taxus* × *media* Hicksii. It was also isolated by Ma et al. from cell cultures of *Taxus baccata*.[5, 37]

Taxane Epoxides

Baccatin I (Compound **48**) and 1β-hydroxybaccatin I (Compound **45**) were both isolated as crystalline compounds. They were identified by NMR spectral data, which were in agreement with published data.

C-14 Oxygenated Taxanes

Compound **49** (2α,5α,9α,10β,14β-pentaacetoxytaxa-4(20),11-diene) was obtained as an amorphous solid. It was identified as a yunnanxane-like (cf. compound **26**, Chapter 3) compound with oxygenation at the C-14 position. Its spectral properties were identical to those published by Yang et al.[72]

A crystalline compound (Compound **50**) with oxygenation at the C-14 position was isolated with a yield of 0.004% (0.95g). The compound crystallized from the ethyl acetate and ligroine to give needles that melt at 155-157°C. The NMR data matches that for taxayunnanine B, which was isolated from the roots of *Taxus yunnanensis* by Zhang et al. No report of the melting point was given by Zhang.[75]

200mg of compound **29**, which was also obtained from the bark (Chapter 3), crystallized from ethyl acetate and ligroine. It was identical to a compound obtained from cell culture of *Taxus chinensis* var. *mairei* by Ma et al.[38]

The structural similarity of compound **51** to compounds **28**, **29**, **49** and **50** is apparent in the ^{1}HNMR and ^{13}CNMR spectra. 30mg of the compound was isolated as an amorphous solid. Spectral analysis shows this compound to be 2α,10β-diacetoxy-5α-hydroxy-14β-(2-methyl)-butyryloxytaxa-4(20),11-diene which is a new member of this class of taxanes. Acetylation of a small amount of compound **51** with acetic anhydride in pyridine gave a compound identical to compound **29** in respect to behavior on TLC. ^{1}HNMR: 0.81 (s, 3H, H-19), 0.86 (s, αMeBu-4'-CH_3), 1.12 (s, 3H, H-17), 1.66 (s, 3H, H-16), 1.82 (d (1.8Hz), 1H, H-1), 2.02 (s, 3H, Ac-CH_3), 2.05 (s, 3H, Ac-CH_3), 2.09 (s, 3H, H-18), 2.82 (dd (9Hz and 18.9Hz), 1H, H-13), 3.24 (d (7.2Hz), 1H, H-3), 4.18 (t (3Hz), 1H, H-5), 4.74 (s, 1H, H-20), 5.05 (dd (4.8Hz and 9Hz), 1H, H-14), 5.10 (s, 1H, H-20), 5.35 (dd (1.8Hz and 7.2Hz), 1H, H-2), and 6.11 (dd (5.7Hz and 12Hz), 1H, H-10). ^{13}CNMR: 11.5, 16.5, 20.8, 21.3, 21.4, 22.2, 25.3, 26.7, 28.8, 31.7, 33.0, 33.8, 37.3, 39.9, 41.0, 43.7, 43.7, 59.3, 70.3, 70.4, 76.0, 113.2, 134.4, 135.9, 147.7, 169.9, 170.2 and 176.0.

Phenanthrene

Phenanthrene crystallized from column fractions and was purified by sublimation. Substituted phenanthrenes are common compounds in plants but phenanthrene ($C_{14}H_{10}$) itself is unusual. An authentic sample of phenanthrene was purified by sublimation. The physical and spectral properties of the crystals from the column were identical to those of phenanthrene. Mass spectrometry returned an M^+ peak of 178.

The presence of phenanthrene in a plant is unusual. Its presence is most likely due to contamination. Plants have been known to take up soil contaminants. Some experiments with food crops grown in contaminated soil have shown that the plants absorb phenanthrene and other hydrocarbons. Alternatively, the plant material may have become contaminated in the grinding process. Grease used to lubricate the machinery may have contained some amount of phenanthrene.

Stearic Acid

Stearic acid ($C_{18}H_{36}O_2$) is a common long-chain fatty acid. The crystals that formed from the fractions were examined by ^{1}HNMR and ^{13}CNMR. The NMR was characteristic of a long-chain saturated fatty acid. After recrystallization from methanol, the melting point was determined as 67-68°C (lit. 69-70°C).

Conclusion

The heartwood of *Taxus brevifolia* does not contain paclitaxel. Vidensek et al. did an HPLC study of various parts of *Taxus brevifolia* to determine levels of paclitaxel. They found an average amount of paclitaxel in the heartwood of 0.0006% (0.015% in the bark).[66] Paclitaxel C, which is present in the wood, is what was most likely measured.

Paclitaxel C is a very potent anticancer agent in its own right (NCI human tumor panel, Ma et al.[37]), but the levels in the wood make its isolation impractical. 10-deactetylpaclitaxel C-7-xyloside however is present in a significant amount (0.013%). 10-deactetylpaclitaxel C-7-xyloside can be converted to paclitaxel C by Rao's xyloside hydrolysis method.[47] 10-Deactetylpaclitaxel C is also present in the wood.

13-Acetyl-7,9-dideacetylbaccatin III, compound **46,** was obtained with a yield of 0.002%. This compound has been isolated from the needles of *Taxus canadensis* and used to make the 9-dihydro analog of paclitaxel, an active analog with better water solubility.

Baccatin IV has not been used to synthesize an active analog of paclitaxel but the potential is there. It was obtained here in a yield of 0.03%. Exhaustive processing of the extract, however, would most likely increase that yield several times. An alteration of the parameters used for the reverse-phase and subsequent silica chromatography on the heartwood extract is necessary. Baccatin IV eluted across too broad a range of mobile phase from the C-18 column. The solvent system for elution should have started at 30% acetonitrile/water rather than 35%. Due to material and time constraints, a second reverse-phase column on the wood extract was never run.

The heartwood of the pacific yew could be used as a source of these important compounds rather than being left behind after the bark is removed.

CHAPTER 6
CHEMISTRY OF SELECTED TAXANES

Introduction

A great deal of work has been done on the chemistry of paclitaxel. Many reviews covering the chemistry and other aspects of paclitaxel have been published. Much of the work in this area has been done by David G. I. Kingston's group.[33, 34] Another review comes from K. C. Nicolaou's group, who tied with Holton's group in publishing the first total synthesis of paclitaxel.[43] Several books have also been published.[12, 64, 65]

These studies depend on the availability of compounds that must be isolated from plant materials. The large-scale isolation of taxanes from plant bio-mass made a number of compounds available in sufficient quantity to study their chemistries. Reactions were carried out on selected taxanes that have been isolated mainly from the bark of *Taxus brevifolia*.

Hydrolysis

The availability of a large amount of compound **12** (Figure 3.1) made it a good candidate for reactivity studies. It was subjected to hydrolysis under basic conditions to determine the sequence in which the ester groups could be hydrolyzed. Hydrolysis, under acidic conditions, was not pursued because the oxetane ring is acid sensitive. The compound was relatively stable to alcoholic ammonia, but in alcoholic potassium carbonate, hydrolysis proceeded more rapidly.[54]

Hydrolysis of compound **12** (Figure 6.1) gave compound **15**, which was also a naturally occurring taxane (see Chapter 3). The acetate at the 10-position was the most labile group for the fully esterified (except for the tertiary hydroxyl at C-15) compound. The hydrolysis was followed by TLC. Compound **15** remained in the reaction mixture only as long as the starting material (Compound **12**) was present. Continued hydrolysis rapidly cleaved the esters at positions 7 and 9 yielding compound **52** (Figure 6.2), which was found to be identical by its spectral properties with a compound described by Appendino et al.[2]

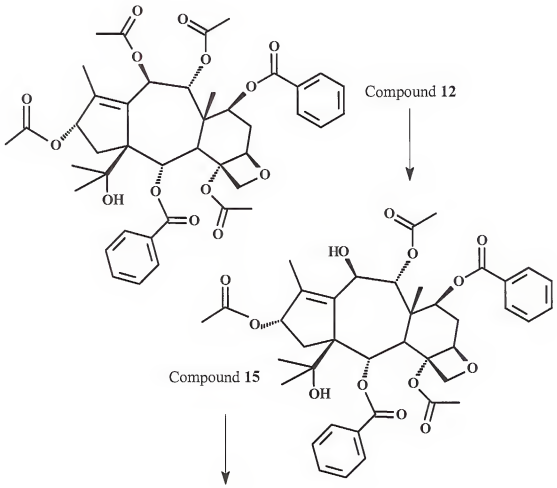


Figure 6.1. Hydrolytic Pathway of Compound **12**

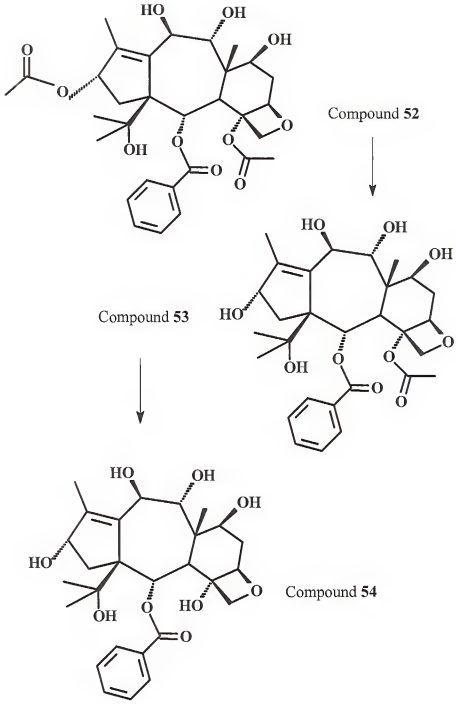


Figure 6.1--continued. Hydrolytic Pathway of Compound **12**

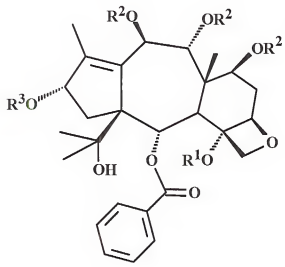
Further hydrolysis cleaved the acetate at the 13-position giving compound **53** (Figure 6.2). The same compound was described by Chen et al. and by Appendino et al.[2, 13] The hydroxyl, at the 13-position, assists in hydrolyzing the 4-acetate due to their proximity (the taxane diterpenoids assume a cupped shape bringing the ends together), which gives compound **54** (Figure 6.2, also described by Appendino et al[2]). The rate of hydrolysis may be increased either by increasing the amount of potassium carbonate or by adding water to the alcoholic carbonate. Continued hydrolysis, even under strongly basic conditions, did not cleave the 2-benzoate.

Hydrolysis of compounds **10-13** was carried out using methanolic potassium hydroxide (0.5 N) for 6 hours at room temperature. Each of the four compounds gave the same single product, compound **54**. These hydrolytic experiments showed that the 2-benzoate was the most stable ester function in the 11(15→1)-abeotaxane ring-system.

Acetylation of either compound **52** or **53**, in acetic anhydride and pyridine, gives compound **55** (Figure 6.2), whose NMR spectral data agreed with those given by Appendino et al.[2] Acetylation of compound **54** gives compound **56** (Figure 6.2), which has a tertiary hydroxyl at the 4-position with the oxetane ring still intact. The proton NMR spectrum of compound **56** showed somewhat rounded peaks, but a COSY spectrum indicated that the 4-hydroxyl remained free and that the oxetane function was present. The spectral properties of compound **56** agreed with those described by Barboni et al.[4] Acetylation at the 4-position could not be effected even under forcing conditions (DMAP and 60°C).

The hydroxyl at C-15 is not close enough to the ester at C-2 to assist in hydrolysis. The hydroxyl at C-1 usually present in the conventional taxane ring-system does aid

hydrolysis. This is born out by hydrolytic experiments done on baccatin IV (Compound **43**), which was isolated from Taxus brevifolia heartwood (Chapter 5).



	R^1	R^2	R^3
52	Ac	H	Ac
53	Ac	H	H
54	H	H	H
55	Ac	Ac	Ac
56	H	Ac	Ac

Figure 6.2. Reaction Products

Baccatin IV (Figure 6.3), in methanol with potassium carbonate, was monitored by TLC. Over time, a slower spot (compound **57**) forms and later an even slower spot (compound **58**). The 2-position on the 6-8-6-ring system (with a hydroxyl at C-1) hydrolyzed first, showing that the 1-OH does assist in hydrolysis. Hydrolysis followed at the 4 and 13 positions, leaving the esters at 7, 9 and 10.

Another example of the lability of the 2-position on the 6-8-6-ring system is Chaudhary's hydrolysis of the 2-benzoate from 2',7-TES protected paclitaxel. Paclitaxel must have the protecting groups because the C-2'-OH likewise assists in hydrolysis of the side-chain. Re-acylation at C-2 with various carboxylic acids gave some compounds that were far more potent than paclitaxel.[10]

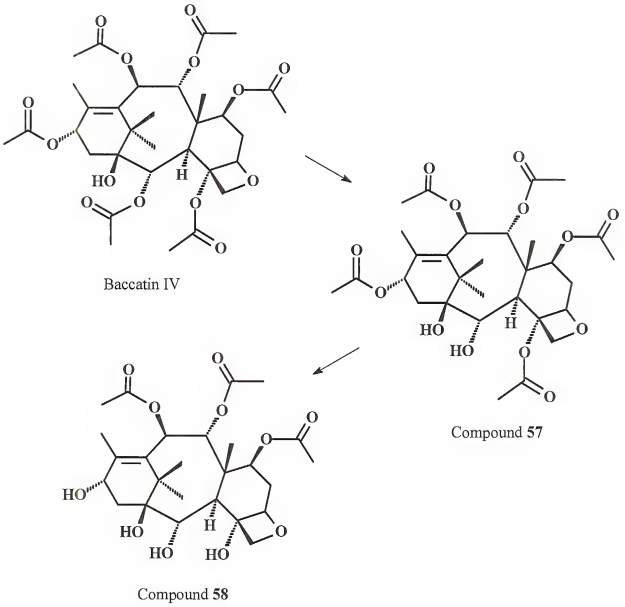


Figure 6.3. Hydrolysis of Baccatin IV

NBS Reactions

Paclitaxel and other taxanes have a tertiary hydroxyl group at the C-1 position. Esters of paclitaxel, at the 1-position, would be beneficial in SAR studies. As demonstrated above, tertiary hydroxyls are resistant to esterification. In an attempt to acylate the tertiary hydroxyl of paclitaxel, a page from the book of protein chemistry was

borrowed. Acyl-hydrazides and N-Bromosuccinimide (NBS) can be used to form peptide bonds. It was observed that benzoylhydrazide and NBS reacted in tertiary-butyl alcohol to cause the esterification of the alcohol, forming *t*-butyl benzoate.

Given the availability of a large quantity of compound **12**, it was chosen as a substrate for the NBS/acyl-hydrazide reaction. Compound **12** has only one hydroxyl, the tertiary hydroxyl at the 15-position. Compound **12**, in dichloromethane, was reacted with 3,5-dimethoxybenzhydrazide (DMBH) and NBS. DMBH was used in preference to benzoylhydrazide because of the ease of detecting its methoxyl groups by NMR. The reaction proceeded very quickly with the destruction of the parent compound. TLC showed the presence of several slower moving compounds. None of these compounds proved to be the ester that was desired. Instead, the oxetane ring of the compound had been opened. Migration of the 4-acetate occurred with ring opening, leading to the mixture of compounds **58** and **59** (Figure6.4).

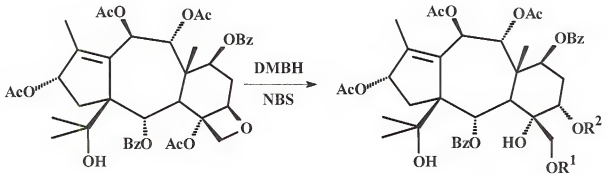


Figure 6.4. Oxetane Ring Opening

Because of the unexpected activity of this reaction, the reaction was carried out on other taxane substrates. Reaction on brevifoliol (Compound **24**) produced compound **60**

(Figure 6.5). Compound **60** had been seen before, in this lab. It was the product of reaction with NBS and silver acetate (or with iodine and silver acetate) on brevifoliol. The 13-hydroxyl attacks at the 20-carbon with migration of the double bond to form a 3(4)-double bond. This also bares out the cupped shape of the taxane ring-system.

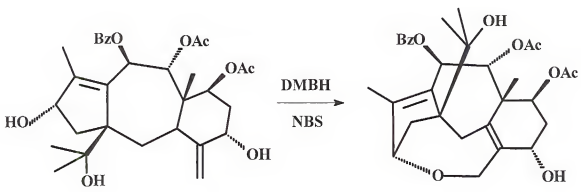


Figure 6.5. Brevifoliol and Reaction Product (Compound **60**)

Reaction on paclitaxel caused contraction of the A-ring but did not open the oxetane ring giving compound **61** (Figure 6.6). Contraction of the A-ring and opening of the oxetane ring have both been observed in paclitaxel, as a product of reaction with electrophilic reagents. Samaranayake, et al prepared **61** by reacting paclitaxel, protected at the 7 and 2'-positions, with mesyl chloride in dichloromethane and triethylamine. It had very good activity in a tubulin depolymerization assay but showed little activity in KB cells.[57]

The NBS/DMBH reaction products from the three compounds above were run in an L1210 cell culture assay using paclitaxel as the standard. None of these products showed activity in this assay.

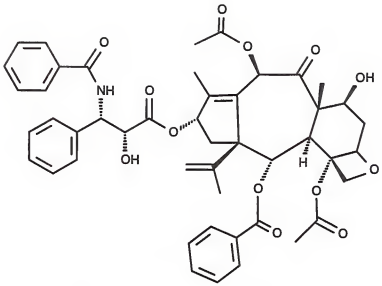


Figure 6.6. A-Nortaxol (Compound **61**)

The mechanism of the NBS/acylhydrazide reaction is difficult to determine. Opening of the oxetane ring and contraction of the A-ring are effects that have been seen with electrophilic reagents. In situ generation of HBr as the reactive species was considered, but reaction with HBr did not give the same results. In addition, the reaction product with brevifoliol is not that which is seen when brevifoliol is reacted with an electrophilic reagent. Reaction of brevifoliol with a Lewis acid, *p*-toluenesulfonic acid, or H_2SO_4 gives compound **62** (Figure 6.7).[14]

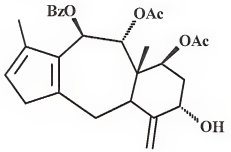


Figure 6.7. Brevifoliol Reaction Product (Compound **62**)

NBS is known to generate free radicals. Free radical reaction is a possible mechanism, but reaction of paclitaxel with AIBN (a free radical generator) in dichloromethane produces only 7-epipaclitaxel. The NBS/acylhydrazide reaction did not epimerize paclitaxel at the 7-position. Therefore, it remains unclear whether the reaction mechanism is due to electrophiles, free radicals, or some other reactive species.

Conclusion

The hydrolytic studies of two compounds having slightly different ring structures shows the great sensitivity to structure that is present in taxane chemistry. Other examples of this sensitivity were also seen in the opening of the oxetane ring and in the rearrangement of the A-ring of paclitaxel. Even brevifoliol, which already possesses the rearranged A-ring and does not have an oxetane ring, exhibits unexpected behavior in reactions.

CHAPTER 7
CONCLUSION

A study started in 1960 to seek out anticancer agents in plants has had some success. One of the most notable compounds to come out of these efforts is paclitaxel. Thirty years elapsed from the collection of tree bark (1962) to the approval by the FDA (1992) of one of the most promising new drugs to treat cancer. Along the way, the study of paclitaxel shed light on a new mechanism for shutting down the growth of cancer cells.

Paclitaxel was obtained in small quantities from the bark of the pacific yew tree (*Taxus brevifolia*). Research into increasing the supply of this important new drug led in several directions: improved methods of isolation, identification of a new source, and synthesis (total or semi-synthesis from a naturally occurring analog). Improved isolation has been the focus of this work. Improved isolation methods are also applicable to obtaining taxanes, such as 10-deacetyl-baccatin III, from which paclitaxel and semi-synthetic derivatives (e.g., docetaxel) can be made.

Paclitaxel and docetaxel are currently the only taxane anticancer agents approved for use. Docetaxel, a semi-synthetic analog of paclitaxel, has some improved characteristics over its naturally occurring cousin such as increased water solubility. It also shows increased activity against some forms of cancer. Other members of this class of drugs, which have shown increased potency over paclitaxel or activity on other cancers, may one-day reach, the clinical stage.

Drugs with much improved characteristics depend on studies which delve into the structure-activity-relationship (SAR) of compounds like paclitaxel. The chemistry of paclitaxel and related taxanes comes into play in modifying the compound at certain sights to determine what, if any effect that change has on activity. As such, it becomes important to analyze what other compounds are present in the plant and determine if they have potential as drugs themselves or as starting materials for semi-synthesis.

Isolation and identification of a drug such as paclitaxel also aids in identifying target sights in a cell where the activity is initiated. Paclitaxel owes its activity to its ability to bind to and precipitate the formation of microtubules from tubulin. Having a substrate to work with allows biochemists to identify the binding site for the drug. This kind of insight into how a drug works can lead to the development of assays which in turn increase the efficiency of developing or finding new drugs which possess that activity.

Several such *in vitro* assays, using tubulin isolated from various sources, have been developed. Tubulin binding and tubulin depolymerization assays have been critical in identifying several new drugs of natural origin that bind at the same sight on tubulin as paclitaxel. By comparing the structures of those compounds, researchers have recently proposed a common pharmacophore responsible for the activity.[45]

The discovery of paclitaxel as an anticancer agent is a lesson in drug development. Research into compounds with related structures led to the development of docetaxel and semi-synthetic paclitaxel. Discerning paclitaxel's mode of action has aided the discovery of non-taxane compounds, which act on tubulin in the same way. New, more potent and cheaper anticancer drugs may soon be developed based on a precious compound from what was once considered a worthless tree.

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BIOGRAPHICAL SKETCH

The author was born to Daniel Martin Juchum and Jacqueline Christine Starriett Juchum on July 15, 1964, in Lompoc, California, the youngest of three brothers. His brothers are Michael Timothy Juchum and Thomas Dean Juchum. He graduated from Spruce Creek High School in Port Orange, Florida, and received a bachelor of science degree in materials science and engineering from the University of Florida in 1989. While studying chemistry in a post-baccalaureate status, he started working for Dr. K. V. Rao at the "Yew of Florida" Taxol Research Project. Dr. Rao later took the author on as a graduate student.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Koppaka V. Rao, Chair (deceased)
Professor of Medicinal Chemistry

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Stephen G. Schulman, Co-Chair
Professor of Medicinal Chemistry

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Kenneth B. Sloan
Professor of Medicinal Chemistry

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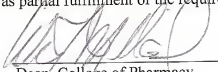
John H. Perrin
Professor of Medicinal Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William R. Dolbier, Jr.
Professor of Chemistry

This dissertation was submitted to the Graduate Faculty of the College of Pharmacy and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1999



Dean, College of Pharmacy

Dean, Graduate School